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(54) Title: TUMOR NECROSIS FACTOR WITH MODIFIED CHANNEL ACTIVITY (57) Abstract An improved form of tumor necrosis factor (TNF) can be employed to regulate TNF channel activity. The identity of the amino acids which line the channel of the TNF molecule and which exert significant control over the activity of the channel are disclosed. The improved form of TNF includes amino acid substitutions, additions, and/or deletions, and/or chemically modified amino acid residues within the channel region for enhancing, diminishing, and/or modulating its channel activity within target membranes. The modified form of TNF is capable of trimerization and of achieving intimate contact with a target membrane containing one or more types of TNF receptor. Contacting target membranes with forms of TNF having modified channel activities can be employed to regulate the permeability and/or response of the target membrane. Greater control over the regulation of the permeability and/or response of target membranes can be achieved with modified forms of TNF as compared to unmodified TNF.		

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DescriptionTUMOR NECROSIS FACTOR WITH MODIFIED CHANNEL ACTIVITY5 Technical Field

The invention relates to modified forms of tumor necrosis factors- α and - β (TNF- α and - β) and related TNF-like molecules. More particularly, the invention relates to modified forms of TNF having enhanced or diminished
10 channel activity as compared to unmodified TNF.

Background Art

Tumor necrosis factor- α and - β (TNF) are polypeptide secretion products produced primarily by activated
15 macrophages and lymphocytes. TNF plays a pivotal role in inflammatory responses, as well as in infectious and neoplastic disease states. TNF- α has been shown to be identical to cachectin. TNF- β is sometimes called lymphotoxin (LT).

20 Human TNF- α is expressed as a 233 residue prohormone and is secreted as a mature protein of 157 residues (17,356 kDa) after cleavage of a 76-amino acid long pro-peptide. The amino acid sequences of several species forms of mature TNF- α are known, viz. human, murine, rat, rabbit, feline,
25 ovine, goat, bovine, and porcine. The amino acid sequence of TNF- α for each of these species is provided in Appendix A. A comparison of the various sequences of TNF- α from the above species indicates that the amino acid sequence of TNF- α is highly conserved, i.e., the sequences for TNF- α
30 from the above non-human species are very similar to the sequence of human TNF- α .

Similarly, human TNF- β is encoded as a 203 residue prohormone and is secreted after cleavage in two forms, i.e. a mature protein of 171 residues (24 kDa) and a mature
35 protein of 148 residues (20 kDa). Mature human TNF- β is glycosylated, but glycosylation is not required for

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activity. The amino acid sequences of several species forms of mature TNF- β are known, viz. human, bovine, murine, and rabbit. The amino acid sequence of the larger mature form of TNF- β for each of these species is provided in Appendix A. A comparison of the various sequences of TNF- β from the above species indicates that the amino acid sequence of TNF- β is highly conserved, i.e., the sequences for TNF- β from the above non-human species are very similar to the sequence of human TNF- β .

It has further been shown by x-ray crystallography that the secreted form of TNF- α consists of three monomers of mature TNF- α associated in the form of a 52,500 kDa trimer (Sprang, S. T. and Eck, M. J., Current Research in Protein Chemistry, Chapter 35: "Subunit Interactions and Function of Tumor Necrosis Factor," pp. 383-394 (1990)). TNF- α is known to bind to at least two forms of TNF receptor and to insert into target membranes. Acidic pH is known to enhance TNF insertion into target membranes. TNF- β has been similarly shown to form trimers and to bind and insert into target membranes. The 3-D structure of the TNF- β trimer is also known (Eck et al., The Journal of Biological Chemistry, 267, 2119-2122 (1992)). Both forms of TNF have been shown to play pivotal roles in inflammatory responses, as well as in infectious and neoplastic disease states.

Disclosure of Invention

Trimers of unmodified TNF are shown to bind and insert into target membranes and to form ion channels therein (Kagan, B. L., Baldwin, R. L., Munoz, D. and Wisnieski, B. J., Science vol xx, pp-pp (1992), "Formation of Ion-Permeable Channels by Tumor Necrosis Factor- α ," incorporated herein by reference).

The invention is a modified form of tumor necrosis factor (TNF). The modified form of TNF may have amino acid substitutions, additions, and/or deletions, and/or

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chemically modified amino acids for enhancing, diminishing, and/or modulating the channel activity of such modified TNF within target membranes. However such enhancement, diminution, and/or modulation of the channel activity occurs while such modified TNF retains a capacity to bind to one or more TNF receptors. Furthermore, such enhancement, diminution, and/or modulation of the channel activity occurs while such modified TNF retains a capacity to trimerize with itself, with other forms of modified TNF, and/or with corresponding forms of unmodified TNF. The invention also includes methods for using such modified forms of TNF.

Preferred modes of achieving the enhancement, diminution, and/or modulation of channel activity include: the enlargement or reduction of the cross-sectional size of the channel for controlling or modulating the cross-sectional size of channel transportants; lining the channel with amino acid residues having a preponderance of positive or negative charges for enhancing or diminishing the channel permeability with respect to molecules having net positive or negative charge and for modulating the transport of neutral molecules as compared to charged molecules; controlling the process of channel formation, including channel dilation and closure. In a preferred embodiment, a trimer is covalently held intact by one or more cysteine-cysteine linkages connecting the individual subunits within the trimer.

The modified form of TNF has a channel activity that is substantially enhanced, diminished, and/or modulated with respect to the channel activity of a corresponding form of unmodified TNF. In a preferred mode for determining the enhancement, diminution, and/or modulation of channel activity of a modified form of TNF, the channel activity of the modified TNF is measured within a black lipid membrane system, described infra.

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X-ray crystallographic studies of trimerized TNF show that the TNF trimer exhibits an approximate three-fold axial symmetry. The symmetry axis of rotation of the trimer defines the approximate center of a channel region.

5 X-ray crystallography also identifies the amino acid residues that line the channel region. Amino acid residues that lie in or adjacent to the channel region are denominated as channel residues. In the preferred mode, the amino acid substitutions, additions, and/or deletions

10 and the chemical modifications of amino acids of the modified TNF are directed to channel residues, i.e., amino acid residues that are shown by x-ray crystallography to lie in or adjacent to the symmetry axis of the trimer.

The modified form of TNF retains an ability to bind to

15 one or more TNF receptors. In a preferred mode for determining the binding activity of the modified TNF with respect to one or more TNF receptors, the binding assay may be performed in an in vitro assay as described infra.

The modified form of TNF undergoes a trimerization

20 reaction similar to that of unmodified TNF. The modified form of TNF may trimerize with itself, with other forms of modified TNF, and/or with unmodified forms of TNF. Preferred means for the ascertainment of such trimerization of TNF include standard in vitro assays such as separation

25 by high performance liquid chromatography (HPLC) on gel exclusion (sizing) columns or by ascertaining the electrophoretic mobility of such TNF on a native gel or by ascertaining its electrophoretic mobility on a denaturing gel after treatment with crossing linking agents.

30

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 (a) is an exploded view in perspective of three monomers or subunits of unmodified TNF, illustrating two background subunits and one foreground subunit, the three

35 TNF subunits being in a dissociated form. The topology of

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the subunits is not intended to be precisely representative.

Fig. 1 (b) is a plan view of the dissociated subunits of TNF shown in Fig. 1 (a), viewed from above.

5 Fig. 2 (a) is a perspective of the two background TNF subunits illustrated in Fig. 1 (a), showing the association of those two subunits of TNF as a dimer and the partial formation of a channel. The foreground subunit is omitted. The topology of this TNF dimer and of the partial formation
10 of the channel is not intended to be precisely representative.

Fig. 2 (b) is a plan view of the background dimer of TNF illustrated in Fig. 2 (a) and of the dissociated foreground subunit illustrated in Fig. 1 (a), viewed from
15 above

Fig. 3 (a) is a perspective of the foreground subunit and of the two background subunits of TNF illustrated in Fig. 1 (a), showing the association of all three subunits of TNF as a trimer. The view of the channel is blocked by
20 the foreground subunit. The topology of this trimer is not intended to be precisely representative.

Fig. 3 (b) is a plan view of the trimer of TNF illustrated in Fig. 3 (a), viewed from above, showing the formation of a channel.

25 Fig. 4 (a) illustrates a sectional view of the trimer of unmodified TNF illustrated in Fig. 3 (a) prior to its insertion into a target membrane. The cross-section passes through the channel of the trimer. The topology of this trimer is not intended to be precisely representative.

30 Fig. 4 (b) is a sectional view of the trimer illustrated in Fig. 4 (a) after such trimer has inserted into a target membrane. Note that the channel dilates after the insertion of the trimer into the target membrane.

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Fig. 5 (a) is a sectional view of a trimer of modified TNF having a channel that is wider than the channel of the unmodified TNF illustrated in Fig. 4 (a).

5 Fig. 5 (b) is a sectional view of the trimer of modified TNF illustrated in Fig. 5 (a) after such trimer has inserted into a target membrane. Note that the wide channel dilates even further after the trimer has inserted into the target membrane.

10 Fig. 6 (a) is a sectional view of a trimer of modified TNF having a channel that is narrower than the channel of the unmodified TNF illustrated in Fig. 4 (a).

15 Fig. 6 (b) is a sectional view of the trimer of modified TNF illustrated in Fig. 6 (a) after such trimer has inserted into a target membrane. Note that the narrow channel of the trimer dilates when the trimer has inserted into the target membrane.

DETAILED DESCRIPTION

20 As illustrated in Fig.'s 3 (a) & (b) and Fig.'s 4 (a) & (b), unmodified TNF monomers (1) form a compact trimer. According to x-ray crystallographic studies, these trimers have an approximate three-fold axis of symmetry, i.e., the structures of the three TNF subunits (1) forming the trimer are not precisely identical to one another. X-ray
25 crystallographic studies also tell us that the trimers have a channel-like structure (2) extending approximately along the axis of symmetry.

Determination of TNF Channel Activity with respect to Neutral Solutes

30 The channel activity of a TNF trimer may be determined by several means. In a preferred method, the pore size and channel activity with respect to uncharged solutes may be determined by the use of multilamellar vesicles systems.
35 Channel activity of the TNF can be correlated with the passive transport of uncharged solutes through the TNF

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channel. Evidence for the passive transport of solutes into a multilamellar vesicle can be derived from observations of the swelling of such multilamellar vesicles. Transport of solutes into a vesicle induces a change in the osmolarity of the fluid in the vesicle lumen. Detection of swelling of the multilamellar vesicles may be obtained by means of light scattering measurements. The rate of swelling will depend upon the activity of the TNF channel with respect to the size of the external solute. Solutes too large to penetrate the channel will not cause the vesicles to swell. The channel activity of unmodified forms of TNF with respect to multilamellar vesicles may be compared with the corresponding channel activity of modified forms of TNF. An observation of differential swelling of multilamellar vesicles with respect to modified and unmodified forms of TNF is a preferred example of a modified "effect" which a modified TNF may have upon a TNF target by virtue of its modified channel activity.

Determination of TNF Channel Activity
with respect to Ions

In an alternative preferred method, the channel activity with respect to ions or other charged molecules, may be ascertained by studying the conductivity of planar membranes (3). Solvent free membranes (3) may be prepared as described by M. Montal, **Methods in Enzymology**, 32, 545 (1974). Squalene (Sigma) or squalane (Fluka) may be employed to coat a hole (100-200 μ m diameter) in a Teflon partition. Monolayers may be spread from mixtures of soybean phosphatidylethanolamin (40%), soybean phosphatidylcholine (40%) (Pelham, AL), and bovine phosphatidylserine (20%) (Avanti). This lipid mixture can also be mixed 1:1 with asolectin (Y. Dagawa and E. Racker, *Journal of Biological Chemistry*, 246, 5477 (1971)). Capacitance measurements may be employed to monitor bilayer formation from the apposition of the two monolayers. After

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membrane formation, the conductance ($g=I/V$) of the unmodified membrane should be ohmic and should lie within the approximate range of 5 to 10 pS. Membranes (3) should be stable within the voltage range of ± 100 mV for at least 10 minutes before the addition of TNF. The aqueous phase should include 100 mM NaCl, 2 mM dimethylglutaric acid (pH 4.5) or 5 mM tris (pH 7.2) as buffer, 2 mM $MgCl_2$, and 1 mM EDTA. Voltage-clamp conditions should be employed. A battery-driven stimulator may be employed to apply voltages and a Keithley 427 current amplifier may be employed to measure current. Output of the current measurement may be displayed on an oscilloscope and recorded on a chart recorder. The cis compartment, to which TNF should be added, is defined as ground. Voltages refer to the trans compartment, opposite the TNF-containing side and analogous to the cytosol of a target cell. In ion selectivity measurements, salt gradients may be imposed across the membrane and the zero-current reversal potential E , where $I = g(V-E)$, may be measured. Silver/silver chloride electrodes may be employed to connect the solutions to the electronics. 3 M KCl/ agar salt bridges may be employed in connection with measurements involving salt gradients.

Upon addition of unmodified TNF to a final concentration of 100 ng/ml, the membrane current I (and therefore conductance, $g=I/V$) remains nearly zero at small voltages (absolute values of $V < 40$ mV). At larger positive voltages ($V > 40$ mV), the current rises within seconds to a new, higher steady state, which is steeply dependent on the membrane voltage. When the polarity of the voltage is reversed, the conductance decreases rapidly to approximately zero. The current remains approximately zero at most negative voltages, whereas current values increase sharply at positive voltages. The conductance induced by TNF is due to the formation of ion-permeable channels. Observed single-channel conductances are heterogeneous, but may be grouped

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into two main classes, viz. one class centered at approximately 5-10 pS, and a second larger class ranging from approximately 100 - 1000 pS. The most frequently observed event is the 5 pS class. Although channels can occasionally form at pH 7.2, channel formation is enhanced by lowering the pH of the aqueous phase containing TNF. The TNF channels exhibit preferential permeability for cations over anions, but not absolutely so. In a tenfold concentration gradient of NaCl, a TNF-treated membrane (3) showed a reversal potential of approximately 25-30 mV. The channel activity of unmodified forms of TNF with respect to planar membranes (3) may be compared with the corresponding channel activity of modified forms of TNF. An observation of differential conductance of planar membranes (3) with respect to modified and unmodified forms of TNF is a preferred example of a modified "effect" which a modified TNF may have upon a TNF target by virtue of its modified channel activity.

Determination of TNF Channel Activity with Cells

In an alternative preferred mode, the channel activity of TNF may be ascertained with respect to cancer cells. The addition of unmodified recombinant human TNF to human U937 histiocytic lymphoma cells increases $^{22}\text{Na}^+$ uptake by 100 to 300%, in the presence or absence of ouabain. Human U937 cells (American Type Culture Collection, ATCC) are washed four times in buffer A (100 mM choline chloride, 25 mM MgCl_2 , 5 mM KCl, and 20 mM Hepes, with the addition of 7.17 mM NaOH to adjust the pH to 7.2) Each sample, containing 2×10^6 cells, may be resuspended in 200 μl of buffer A after the last wash and equilibrated at 4°C for 15 minutes before the addition of 1 μg TNF (2 μl of a 0.5 mg/ml stock in 10 mM sodium phosphate and 0.2 M NaCl; pH 7) or 2 μl of buffer alone. Binding is allowed to proceed for 2 hours at 4°C. Then 10 μl of 20 mM ouabain in water or 10

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μ l of water alone may be added, and the samples incubated for 13 minutes at 37°C. Then, 10 μ l of $^{22}\text{NaCl}$ [10 μM stock, 200 $\mu\text{Ci/ml}$ (Amersham)] may be added to each sample, and incubation at 37°C may be continued for 10 minutes. Ice-cold PBS (0.8 ml; 10 mM sodium phosphate and 150 mM NaCl) may be added to stop the flux of $^{22}\text{Na}^+$. The cells may then be pelleted in a microcentrifuge (Bechman) and washed twice with 1 ml of PBS. Aliquots (10 μ l) of the first and last supernatants are then removed for counting. Pelleted cells may be solubilized by incubation for 15 minutes with 100 μ l of 0.5% Triton X-100 in buffer A at 37°C. Solubilized cells and supernatant aliquots may then be mixed with 10 ml of liquid scintillant and counted at the ^{14}C setting of a Beckman scintillation counter. Na^+ uptake values may be based on the presence of 9.365 mM Na^+ (radioactive plus cold). This assay is a modification of that of J.B. Smith and E. Rozengurt, **Proceedings of the National Academy of Sciences, U.S.A.**, 75, 5560 (1978). The channel activity of unmodified forms of TNF with respect to cancer cells may be compared with the corresponding channel activity of modified forms of TNF. An observation of differential sodium ion permeability of human U937 histiocytic lymphoma cells with respect to modified and unmodified forms of TNF is a preferred example of a modified "effect" which a modified TNF may have upon a TNF target by virtue of its modified channel activity.

Binding Assay with respect to

TNF Target

A binding assay may be performed as described by Loetscher et al. (*Cell*, 61, 351-359 (1990)), as modified herein. The binding assay may be performed with any of a large number of naturally occurring human cell types which express one or more of the TNF receptors. Alternatively, the binding assay may be performed with model cells such as human U937 histiocytic lymphoma cells or with COS-1 cells

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that have been transfected with the 1.3 kb gene for the 55 kDa TNF receptor. After 2-3 days in culture, the transfected COS-1 cells may be detached with EDTA and tested for binding by ^{125}I -TNF- α or ^{125}I -TNF- β . The cells are washed, resuspended at 2.8×10^6 cells/milliliter, and incubated with various concentrations of ^{125}I -TNF- α or ^{125}I -TNF- β in the absence and presence of a 500-fold excess of cold TNF- α or TNF- β , respectively, for 2 hours at 4°C. The bound radioactivity is then counted in a gamma counter. Nonspecific binding is subtracted to obtain the net specific binding of the ^{125}I -TNF- α or ^{125}I -TNF- β to the transfected COS-1 cells. An alternative method for performing the binding assay is described by Coffman et al. (Lymphokine Research, 7, 371-383 (1988)).

Detection of Trimerization

Both modified and unmodified forms of TNF may undergo a trimerization reaction. The modified form of TNF may trimerize with itself, with other forms of modified TNF, and/or with unmodified forms of TNF. Preferred means for the ascertainment of such trimerization of TNF include standard in vitro assays such as separation of the monomer and trimer forms by high performance liquid chromatography (HPLC) on gel exclusion (sizing) columns. Alternatively, the sizes of modified and unmodified forms of TNF may be ascertained from their electrophoretic mobilities on a native gel or they may be ascertained by contrasting their electrophoretic mobilities on a denaturing gel after treatment with cross-linking agents.

SUBSTITUTIONS FOR TNF- α

An amino acid sequence 157 residues long for an active form of human TNF- α is listed in Appendix A. Other forms of human TNF- α may have a greater or lesser number of amino acid residues, i.e. there may be deletions or additions at either end of the peptide sequence. Furthermore, there are

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a number of muteins of human TNF- α having one or more substitutions, deletions, and/or additions. However, the numbering of the amino acid sequence of all such alternate forms of human TNF- α may be adapted so as to correspond to the number of the sequence for human TNF- α given in Appendix A.

Also listed in Appendix A are amino acid sequences for TNF- α from 8 other species, including porcine, bovine, goat, ovine, feline, rabbit, rat and murine. Some forms of non-human TNF- α are known to be glycosylated, but glycosylation is not required for activity. It can be anticipated that TNF- α will be found in further species as well and will be similarly sequenced.

There is a close sequence homology between human TNF- α and TNF- α from non-human sources. Where the total number of amino acids within a sequence of a non-human TNF- α is less than the total number of amino acids within human TNF- α , one or more blank insertions have been introduced into the sequence numbering of the non-human TNF- α . The insertions of such blanks into the sequence numbering of non-human forms of TNF- α is done in such a way so as to maximize the sequence homology between human TNF- α and non-human TNF- α . The sequence numbering found in Appendix A and employed herein with respect to non-human forms of TNF- α is that sequence numbering that yields the greatest sequence homology between each non-human form of TNF- α and human TNF- α .

As indicated in the assay described above, TNF- α can be shown to have channel activity when it is inserted into a planar lipid membrane (3). With respect to human TNF- α , the amino acid residues that line or face this channel, i.e. the primary "channel residues," include the following, viz.: Lys¹¹, Leu⁵⁷, Tyr⁵⁹, Lys⁹⁸, Lys¹¹², Glu¹¹⁶, Tyr¹¹⁹, Gly¹²¹, Ile¹⁵⁵, and Leu¹⁵⁷. However, these primary "channel residues" are encompassed within a larger group of amino acids designated as channel liners, some of which merely contact

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the channel via the carbonyl oxygens and amide NH groups of the peptide chain. For TNF- α these channel liners include residues number 11, 55-59, 98-125, and 151-157. Because of the close sequence homology between human TNF- α and non-human forms of TNF- α , the important channel residues with respect to non-human forms of TNF- α have the same sequence numbers as given above for human TNF- α . It can be anticipated that, of those mutein forms of human and non-human TNF- α that exhibit modified channel activity, the important channel residues for such mutein forms of TNF- α will also have the same sequence numbers as given above for human TNF- α . However, the particular amino acids that occupy such sequence sites may vary from one non-human species to the next or from one mutein form to another.

Listed below are preferred amino acid substitutions with respect to channel residues of TNF- α for enhancing, diminishing, or modulating its channel activity. In each instance, the indicated parent amino acid is the amino acid found in human TNF- α with respect to that particular sequence number. The parent amino acid with respect to non-human forms of TNF- α and with respect to various mutein forms of TNF- α may differ from the indicated parent amino acid. In such instances, the parent amino acid may be determined by referring to the sequence number for the particular channel residue being substituted.

Preferred amino acid substitutions of channel residues with respect to TNF- α include the following:

1. Residue No.: 11 Lys

First preference: Glu, Arg, Cys, Asp, Gln, Asn, Ser, Thr, & His

Second Preference: Val, Leu, Ile, & Ala

Third Preference: Trp, Gly, Pro, Tyr, Phe, & Met

2. Residue No.: 57 Leu

First preference: Trp, Ser, Thr, Ala, Met, Cys, Phe, & Tyr

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- Second Preference: Arg, Glu, Lys, Asp, Gln, & Asn
- Third Preference: Gly, Val, Ile, His, & Pro
3. Residue No.: 59 Tyr
- 5 First preference: Trp, Ser, Thr, Ala, Met, & Cys
- Second Preference: Arg, Glu, Lys, Asp, Gln, & Asn
- Third Preference: Gly, Val, Ile, Leu, His, & Pro
- 10 4. Residue No.: 98 Lys
- First preference: Arg, Cys, Glu, Asp, Gln, Asn, Ser, Thr, & His
- Second Preference: Val, Leu, Ile, & Ala
- 15 Third Preference: Trp, Met, Gly, Pro, Tyr, & Phe
5. Residue No.: 112 Lys
- First preference: Arg, Cys, Asp, Gln, Asn, Ser, Thr, Glu, & His
- Second Preference: Val, Leu, Ile, & Ala
- 20 Third Preference: Trp, Gly, Pro, Tyr, & Phe
6. Residue No.: 116 Glu
- First preference: Lys, Arg, Cys, Asp, Gln, Asn, Ser, His, & Thr
- Second Preference: Leu, Ile, & Ala
- 25 Third Preference: Trp, Met, Gly, Pro, Tyr, & Phe
7. Residue No.: 119 Tyr
- First preference: Trp, Phe, Ser, Thr, Ala, Met, & Cys
- 30 Second Preference: Arg, Glu, Lys, Asp, Gln, & Asn
- Third Preference: Gly, Val, Ile, Leu, & Pro
8. Residue No.: 121 Gly
- First preference: Ala, Val, Ser, & Thr
- 35 Second Preference: Pro, Ile, Leu, & His

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Third Preference: Trp, Tyr, Phe, Cys, Met, Lys,
Glu, Arg, Gln, Asp, & Asn

9. Residue No.: 155 Ile

First preference: Trp, Ser, Thr, Ala, Met, Cys,
Phe, & Tyr

5

Second Preference: Arg, Glu, Lys, Asp, Gln, &
Asn

Third Preference: Gly, Val, His, & Pro

10. Residue No.: 157 Leu

10

First preference: Trp, Ser, Thr, Ala, Cys, &
Tyr

Second Preference: Arg, Glu, Lys, Asp, & Asn

Third Preference: Gly, Ile, & His

15

SUBSTITUTIONS FOR TNF- β

An amino acid sequence 171 residues long for an active form of human TNF- β (lymphotoxin) is listed in Appendix A. Other forms of human TNF- β may have a greater or lesser number of amino acid residues, i.e. there may be deletions or additions at either end of the peptide sequence. Furthermore, there are a number of muteins of human TNF- β having one or more substitutions, deletions, and/or additions. However, the numbering of the amino acid sequence of all such alternate forms of human TNF- β may be adapted so as to correspond to the numbering of the sequence for human TNF- β given in Appendix A.

Also listed in Appendix A are the amino acid sequences for TNF- β from 3 other species, including bovine, rabbit, and murine. It can be anticipated that TNF- β will be found in further species as well and will be similarly sequenced.

There is a close sequence homology between human TNF- β and non-human TNF- β . Where the total number of amino acids within a sequence of non-human TNF- β is less than the total number of amino acids within human TNF- β , one or more blank insertions have been introduced into the sequence numbering

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of the non-human form of TNF- β . Such insertions are positioned so as to maximize the sequence homology between human TNF- β and non-human TNF- β . The sequence numbering found in Appendix A and employed herein with respect to non-human forms of TNF- β is that sequence numbering that yields the greatest sequence homology between such non-human forms of TNF- β and human TNF- β .

TNF- β can be shown to have channel activity when it is inserted into a planar lipid membrane (3). With respect to human TNF- β , the amino acid residues that line or face this channel, i.e. the "channel residues," include the following, viz.: Lys²⁸, Phe⁷⁴, Tyr⁷⁶, Lys¹¹⁹, Glu¹²⁷, His¹³¹, Tyr¹³⁴, Gly¹³⁶, Phe¹⁶⁹, and Leu¹⁷¹. However, these primary "channel residues" are encompassed within a larger group of amino acids designated as channel liners, some of which merely contact the channel via the carbonyl oxygens and amide NH groups of the peptide chain. For TNF- β these channel liners include residues number 28, 72-76, 119-140, and 165-171. Because of the close sequence homology between human TNF- β and non-human forms of TNF- β , the important channel residues with respect to non-human forms of TNF- β have the same sequence numbers as given above for human TNF- β . It can be anticipated that, of those mutein forms of human and non-human TNF- β that exhibit altered channel activity, the important channel residues for such mutein forms of TNF- β will also have the same sequence numbers as given above for human TNF- β . However, the particular amino acids that occupy such sequence sites may vary from one non-human species to the next or from one mutein form to another.

Listed below are preferred amino acid substitutions with respect to channel residues of TNF- β for enhancing, diminishing, or modulating its channel activity. In each instance, the indicated parent amino acid is the amino acid found in human TNF- β with respect to that particular sequence number. The parent amino acid with respect to

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non-human forms of TNF- β and with respect to various mutein forms of TNF- β may differ from the indicated patent amino acid. In such instances, the parent amino acid may be determined by referring to the sequence number for the particular channel residue being substituted.

Preferred amino acid substitutions of channel residues with respect to TNF- β include the following:

1. Residue No.: 28 Lys
 - First preference: Glu, Arg, Cys, Asp, Gln, Asn, Ser, Thr, & His
 - Second Preference: Val, Leu, Ile, & Ala
 - Third Preference: Trp, Gly, Pro, Tyr, Phe, & Met
2. Residue No.: 74 Phe
 - First preference: Trp, Ser, Thr, Ala, Met, Cys, Leu, & Tyr
 - Second Preference: Arg, Glu, Lys, Asp, Gln, & Asn
 - Third Preference: Gly, Val, Ile, His, & Pro
3. Residue No.: 76 Tyr
 - First preference: Trp, Ser, Thr, Ala, Met, Cys, & Phe
 - Second Preference: Arg, Glu, Lys, Asp, Gln, & Asn
 - Third Preference: Gly, Val, Ile, Leu, His, & Pro
4. Residue No.: 119 Lys
 - First preference: Arg, Cys, Glu, Asp, Gln, Asn, Ser, Thr, & His
 - Second Preference: Val, Leu, Ile, & Ala
 - Third Preference: Trp, Met, Gly, Pro, Tyr, & Phe
5. Residue No.: 127 Glu
 - First preference: Arg, Cys, Asp, Gln, Asn, Ser, Thr, Lys, & His
 - Second Preference: Val, Leu, Ile, Met, & Ala

- 18 -

- Third Preference: Trp, Gly, Pro, Tyr, & Phe
6. Residue No.: 131 His
 First preference: Lys, Arg, Cys, Asp, Gln, Asn, Ser, Glu, & Thr
 5 Second Preference: Leu, Ile, Val, & Ala
 Third Preference: Trp, Met, Gly, Pro, Tyr, & Phe
7. Residue No.: 134 Tyr
 First preference: Trp, Phe, Ser, Thr, Ala, Met, & Cys
 10 Second Preference: Arg, Glu, Lys, Asp, Gln, His, & Asn
 Third Preference: Gly, Val, Ile, Leu, & Pro
8. Residue No.: 136 Gly
 First preference: Ala, Val, Ser, & Thr
 15 Second Preference: Pro, Ile, Leu, & His
 Third Preference: Trp, Tyr, Phe, Cys, Met, Lys, Glu, Arg, Gln, Asp, & Asn
9. Residue No.: 169 Phe
 First preference: Trp, Ser, Thr, Ala, Met, Cys, Ile, & Tyr
 20 Second Preference: Arg, Glu, Lys, Asp, Gln, Leu, & Asn
 Third Preference: Gly, Val, His, & Pro
- 25 10 Residue No.: 171 Leu
 First preference: Trp, Ser, Thr, Ala, Cys, Phe, & Tyr
 Second Preference: Arg, Glu, Lys, Asp, Gln, & Asn
 30 Third Preference: Gly, Pro, Val, Ile, Met, & His

MECHANISMS WHEREBY CHANNEL
ACTIVITY MAY BE CONTROLLED

- 35 The channel activity of unsubstituted forms of TNF- α and - β share many broad similarities. Similarly, there is

- 19 -

a correspondence between the specific channel residues of TNF- α and - β . The correspondence between the channel residues of human TNF- α and - β is as follows:

	TNF- α		TNF- β
5	Lys ¹¹	corresponds to	Lys ²⁸
	Leu ⁵⁷	corresponds to	Phe ⁷⁴
	Tyr ⁵⁹	corresponds to	Tyr ⁷⁶
	Lys ⁹⁸	corresponds to	Lys ¹¹⁹
	Lys ¹¹²	corresponds to	Glu ¹²⁷
10	Glu ¹¹⁶	corresponds to	His ¹³¹
	Tyr ¹¹⁹	corresponds to	Tyr ¹³⁴
	Gly ¹²¹	corresponds to	Gly ¹³⁶
	Ile ¹⁵⁵	corresponds to	Phe ¹⁶⁹
	Leu ¹⁵⁷	corresponds to	Leu ¹⁷¹

15 The above list of channel residues for TNF- α and - β are correlated because these channel residues are similarly positioned within their respective channels and because an amino acid substitution of correlated channel residues tends to cause correlated changes of channel activity with
 20 respect to both TNF- α and - β . For example a substitution of a long chain aliphatic for Gly¹²¹ (TNF- α) and Gly¹³⁶ (TNF- β) will tend to occlude the channel and will tend to diminish the channel activity of both TNF- α and - β . Similarly, the removal of bulky amino acids and the
 25 substitution of short chain amino acids tends to broaden the cross-sectional diameter of the channel and frequently causes an increase in channel activity.

30 Amino Acid Substitutions which Employ
 Cysteine-Cysteine Bonding to
 Cross-link Subunits within a TNF Trimer

 X-ray crystallographic studies on the trimers of both TNF- α and - β indicate the usage of several salt bridges to bond together the respective TNF subunits. The amino acid
 35 residues for these salt linkages can be substituted with

- 20 -

cysteines in order to form covalent cysteine-cysteine linkages between respective TNF subunits.

Examples for TNF- α include the following:

- 5 A. The interchain salt linkage between Lys⁹⁸ of one subunit and Glu¹¹⁶ of an adjacent subunit can be modified by substituting Cys for both residues #98 and #116. When the resulting modified form of TNF trimerizes under non-reducing conditions, three interchain covalent linkages will form, viz. the Cys⁹⁸ of each subunit will form a covalent disulfide bond with the Cys¹¹⁶ on the adjacent subunit. The resultant disulfide bond can be disrupted under reducing conditions.
- 10 B. Similarly, the interchain salt linkage between Arg¹⁰³ of one TNF subunit and Glu¹⁰⁴ of an adjacent TNF subunit may be modified by Cys substitutions to form a modified form of TNF having Cys¹⁰³ and Cys¹⁰⁴ to form three interchain disulfide bonds for stabilizing the TNF trimer.
- 15 C. Similarly, the interchain salt linkage between Lys¹¹ of one TNF subunit and the carboxy terminal Leu¹⁵⁷ of an adjacent TNF subunit may be modified by Cys substitutions to form a modified form of TNF having Cys¹¹ and Cys¹⁵⁷ to form three interchain disulfide bonds for stabilizing the TNF trimer.
- 20 D. The Tyr¹¹⁹ residues that protrude into the middle of the TNF channel can be substituted with Cys¹¹⁹. However, in this case no salt bridge is replaced and only one interchain disulfide bond results under non-reducing conditions. However, this single disulfide bond may switch from one subunit pair to another, e.g., from TNF1-SS-TNF2 to TNF2-SS-TNF3 to TNF3-SS-TNF1.
- 25
- 30

Examples for TNF- β include the following:

- 35 A. A pH dependent interchain salt linkage between Lys¹¹⁹ of one subunit and His¹³¹ of an adjacent

- 21 -

- subunit can be modified by substituting Cys for both residues #119 and #131. When the resulting modified form of TNF trimerizes under non-reducing conditions, three interchain covalent linkages will form, viz. the Cys¹¹⁹ of each subunit will form a covalent disulfide bond with the Cys¹³¹ on the adjacent subunit. The resultant disulfide bond can be disrupted under reducing conditions, but, unlike the salt bridge, will be relatively independent of pH.
- 5
- 10
- 15
- 20
- 25
- 30
- B. Similarly, the interchain linkage between Ser¹¹⁷ of one TNF subunit and His¹³⁵ of an adjacent TNF subunit may be modified by Cys substitutions to form a modified TNF having Cys¹¹⁷ and Cys¹³⁵ to form three interchain disulfide bonds for stabilizing the TNF trimer.
- C. Similarly, the interchain salt linkage between Lys²⁸ of one TNF subunit and the carboxy terminal Leu¹⁷¹ of an adjacent TNF subunit may be modified by Cys substitutions to form a modified form of TNF having Cys²⁸ and Cys¹⁷¹ to form three interchain disulfide bonds for stabilizing the TNF trimer.
- D. The Tyr¹³⁴ residues can be substituted with Cys¹³⁴. However, in this case no salt bridge is replaced and only one interchain disulfide bond results under non-reducing conditions. However, it is possible that this single disulfide bond may switch from one subunit pair to another, e.g., from TNF1-SS-TNF2 to TNF2-SS-TNF3 to TNF3-SS-TNF1.

Chemical Modification of TNF Channel for
Diminishing or Modulating Channel Activity

A number of reagents may be employed to interact selectively with channel residues of modified and unmodi-

35

- 22 -

fied forms of TNF so as to form an occlusion within the channel for diminishing its activity.

5 A Method for Making Modified Forms of
 TNF- α and/or - β Having a Reconstructed Channel
 with One or More Amino Acid Substitutions,
 As Compared to Unmodified TNF- α and/or - β
 For Regulating Channel Activity

10 Firstly, one or more candidate forms of modified TNF- α
and/or - β are formed by substituting one or more channel
residues with replacement amino acids. The preferred
channel residues and the respective preferred amino acid
substitutions for such channel residues are indicated
above.

15 A preferred mode for effecting such amino acid substi-
tutions by means of site-specific mutagenesis is described
by Kunkel, T. A.. (**Proceedings of the National Academy of**
Sciences, U.S.A., 82, 488-492). Kunkel teaches that plas-
mid pHTP320 may be digested with **SpeI** and **HindIII** to iso-
20 late the DNA fragment containing the TNF gene, and may be
subcloned into the **HindIII** and **XbaI** sites of phage M13mp19.
From this recombinant phage, single-stranded DNA may be
prepared as a template containing uracils for mutagenesis
by using **E. coli CJ236**. Appropriate mutagenic
25 oligonucleotides (approximately 20-mers) corresponding to
the desired amino acid substitution may be chemically
synthesized with a DNA synthesizer. For each mutagenesis,
approximately 200 ng (0.1 pmol) of a template containing
uracil may be mixed with 4 pmol of 5'-phosphorylated
30 mutagenic oligonucleotide in 10 μ l of an annealing buffer
(20 mM Tris-HCl, pH 7.4; 2 mM MgCl₂; 50 mM NaCl). The
reaction mixture may be heated at 70°C for 10 minutes, and
then cooled at a rate of approximately 1°C/min. until 30°C.
After addition of DNA polymerase, Klenow fragment (2.5
35 units), T4 DNA ligase (5 units) and 1 μ l of 10x synthesis
buffer (5mM each dNTP; 10mM ATP; 100 mM Tris-HCL, pH 7.4;

- 23 -

50 mM MgCl₂; 20 mM dithiothreitol), the reaction mixture may be sequentially incubated at 0°C for 5 minutes, 25°C for 5 minutes and 37°C for 90 minutes. A sample of the ligation reaction may be employed to transform competent *E. coli* JM105 cells. Without previous selection by hybridization assay with the mutated oligonucleotide, single-stranded DNA may be extracted from the plaques and identified by nucleotide sequencing. The replicative form of the mutant may be digested with restriction endonucleases **ClaI** and **HindIII**. The fragment containing the mutagenized TNF coding sequence may be subcloned into an expression plasmid pHTP320 in place of the TNF gene. The transformants having an expression plasmid for the modified form of TNF may be incubated in LB medium supplemented with ampicillin (50 µg/ml) at 37°C overnight and then the cultures inoculated in M9 medium supplemented with 0.5% casamino acids and ampicillin (50 µg/ml). After 3 hours, 3-indoleacrylic acid (20 µg/ml) may be added to induce the *E. coli* **trp** promoter and cultivation may be further continued at 37°C for 20 hours. Cells may then be disrupted by lysozyme-digestion and freezing-thawing as described by Nagata et al. **Nature**, 284, 316-320 (1980). The disrupted cells may then be centrifuged to obtain a clarified supernatant as *E. coli* extracts. The candidate TNF may then be purified from the *E. coli* extract according to the method of Yamada et al., **Journal of Biotechnology**, 3, 141-153 (1985).

The purified form of candidate TNF may then tested to determine if it retains an ability to form TNF trimers. The protocol for testing trimerization is given above.

The purified form of candidate TNF may also be tested to determine if the TNF has an ability to achieve intimate contact with a target that includes both a membrane (3) and one or more TNF receptors. The protocols for determining TNF receptor binding and channel formation are given above.

- 24 -

The purified form of candidate TNF may also be tested to determine if the TNF, when in intimate contact with the target, achieves a modified effect. Examples of such modified effects are given above, however, they must be of a type caused by formation of a modified channel activity. The modified channel activity must materially differ from corresponding unmodified channel activities caused by the corresponding unmodified TNF.

A modified form of TNF may be selected from one or more of the candidate forms of TNF. The modified form of TNF must have been determined to be able to form TNF trimers; it must be able to achieve intimate contact with the target; and it must be able to achieve a modified effect upon a target by virtue of a modified channel activity.

After the modified form of TNF is selected, it may be made in purified form and in commercial quantities.

Method for Regulating the Channel Activity
with respect to a Target Membrane

The permeability exhibited by a TNF target membrane (3) may be regulated by contacting the target membrane with a modified form of TNF. The modified form of TNF should have a reconstructed channel (5 or 7), as compared to unmodified TNF, as described above, for regulating channel activity. In a preferred mode, modified form of TNF (6) is selected that forms a reconstructed channel (7) so as to result in a reduction of the channel activity as compared to the unmodified form of TNF. Accordingly, the insertion of such modified form of TNF in the target membrane (3) serves to reduce the channel activity. In an alternative preferred mode, a modified form of TNF (4) is selected that forms a reconstructed channel (5) so as to result in an enhancement of the channel activity as compared to the unmodified form of TNF, as indicated above. Accordingly,

- 25 -

the insertion of this form of modified TNF in the target membrane (3) serves to enhance the channel activity.

Method for Inhibiting the Binding
of Unmodified TNF to one or more TNF Receptors
Attached to a Target Membrane

5 A modified form of TNF having a reconstructed channel (7) for reducing channel activity within a target membrane (3) may be employed for inhibiting the binding of
10 unmodified TNF to one or more TNF receptors attached to a target membrane (3). In a preferred mode, a modified form of TNF having a channel reconstructed for reducing channel activity, as compared to unmodified TNF, is contacted with one or more of TNF receptors under conditions for
15 permitting binding between the modified form of TNF and the TNF receptor. The modified form of TNF acts as an antagonist or competitive inhibitor of the unmodified TNF with respect to binding to TNF receptor.

Regulation of Channel Activity by
Channel Blockers and Activators

20 Molecules may be designed to interact specifically with the channel region of modified and unmodified forms of TNF. Some interactions will occlude the channel and block
25 its channel activity. Some interactions will dilate the channel and enhance its channel activity.

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APPENDIX A

SEQUENCE LISTING FOR TUMOR NECROSIS FACTOR

5 (1) GENERAL INFORMATION:

(i) APPLICANT: Wisnieski, Bernadine J.

10 (ii) TITLE OF INVENTION: Tumor Necrosis Factor with Modified
Ion Channel

(iii) NUMBER OF SEQUENCES: 13

15 (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Donald G. Lewis

(B) STREET: 8328 Regents Road #1E

20 (C) CITY: San Diego

(D) STATE: California

(E) COUNTRY: USA

25 (F) ZIP: 92122

(v) COMPUTER READABLE FORM:

30 (A) MEDIUM TYPE: Diskette, 3.5 inch, 1.44 M storage

(B) COMPUTER: VE System 386

35 (C) OPERATING SYSTEM: MS-DOS 5

(D) SOFTWARE: Word Perfect

(vi) CURRENT APPLICATION DATA:

40 (A) APPLICATION NUMBER: unknown

(B) FILING DATE: 12 March 1993

45 (C) CLASSIFICATION: unknown

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/852,625

50 (B) FILING DATE: 12 March 1992

(viii) ATTORNEY/AGENT INFORMATION:

- 27 -

(A) NAME: Donald G. Lewis

(B) REGISTRATION NUMBER: 28636

(C) REFERENCE/DOCKET NUMBER: BJW-2

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (619) 554-2421

(B) TELEFAX: (619) 554-6312

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 157 amino acids

(B) TYPE: amino acids

(C) TOPOLOGY: linear

(ii) MOLECULAR TYPE: protein

(ix) FEATURE:

(A) NAME/KEY: Tumor Necrosis Factor (human)

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Pennica D., Nedwin G. E., Hayflick, J.S., Seeburg P.H., Derynck, R., Palladino, M.A., Kohr, W.J., et al.

(B) TITLE: Human Tumor Necrosis Factor: Precursor Structure, Expression and Homology to Lymphotoxin

(C) JOURNAL: Nature

(D) VOLUME: 312

(E) PAGES: 724-729

(F) DATE: 1984

(G) RELEVANT RESIDUES IN SEQ ID NO:1: 1-157

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His

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		5		10		15
	Val Val Ala Asn Pro	20	Gln Ala Glu Gly Gln	25	Leu Gln Trp Leu Asn	30
5	Arg Arg Ala Asn Ala	35	Leu Leu Ala Asn Gly	40	Val Glu Leu Arg Asp	45
	Asn Gln Leu Val Val	50	Pro Ser Glu Gly Leu	55	Tyr Leu Ile Tyr Ser	60
10	Gln Val Leu Phe Lys	65	Gly Gln Gly Cys Pro	75	Ser Thr His Val Leu	80
	Leu Thr His Thr Ile	85	Ser Arg Ile Ala Val	90	Ser Tyr Gln Thr Lys	95
	Val Asn Leu Leu Ser	100	Ala Ile Lys Ser Pro	105	Cys Gln Arg Glu Thr	110
20	Pro Glu Gly Ala Glu	115	Ala Lys Pro Trp Tyr	120	Glu Pro Ile Tyr Leu	125
	Gly Gly Val Phe Gln	130	Leu Glu Lys Gly Asp	135	Arg Leu Ser Ala Glu	140
25	Ile Asn Arg Pro Asp	145	Tyr Leu Asp Phe Ala	150	Glu Ser Gly Gln Val	155
	Tyr Phe Gly Ile Ile	155	Ala Leu			

(3) INFORMATION FOR SEQ ID NO:2:

35

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 157 amino acids

40

(B) TYPE: amino acids

(C) TOPOLOGY: linear

(ii) MOLECULAR TYPE: protein

45

(ix) FEATURE:

(A) NAME/KEY: Tumor Necrosis Factor (porcine)

50

(B) OTHER INFORMATION: A blank residue designated by "Xaa" is inserted after residue No. 7 of porcine TNF and the sequence numbering is augmented by 1

- 29 -

starting with residue No. 8 in order to maximize the sequence homology with human TNF.

- 5 (x) PUBLICATION INFORMATION:
- (A) AUTHORS: Pauli, U. Beutler, B., and Peterhans, S.
- 10 (B) TITLE: Porcine Tumor Necrosis Factor- α : Cloning with the Polymerase Chain Reaction and Determination of the Nucleotide Sequence
- (C) JOURNAL: Gene
- 15 (D) VOLUME: 81
- (E) PAGES: 185-191
- (F) DATE: 1989
- 20 (G) RELEVANT RESIDUES IN SEQ ID NO:2: 1-157 (includes one blank)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

25	Leu Arg Ser Ser Ser	Gln Thr Xaa Ser Asp	Lys Pro Val Ala His
	5	10	15
	Val Val Ala Asn Val	Lys Ala Glu Gly Gln	Leu Gln Trp Gln Ser
30	20	25	30
	Gly Tyr Ala Asn Ala	Leu Leu Ala Asn Gly	Val Lys Leu Lys Asp
	35	40	45
35	Asn Gln Leu Val Val	Pro Thr Asp Gly Leu	Tyr Leu Ile Tyr Ser
	50	55	60
	Gln Val Leu Phe Arg	Gly Gln Gly Cys Pro	Ser Thr Asn Val Phe
	65	70	75
40	Leu Thr His Thr Ile	Ser Arg Ile Ala Val	Ser Tyr Gln Thr Lys
	80	85	90
	Val Asn Leu Leu Ser	Ala Ile Lys Ser Pro	Cys Gln Arg Glu Thr
45	95	100	105
	Pro Glu Gly Ala Glu	Ala Lys Pro Trp Tyr	Glu Pro Ile Tyr Leu
	110	115	120
50	Gly Gly Val Phe Gln	Leu Glu Lys Asp Asp	Arg Leu Ser Ala Glu
	125	130	135
	Ile Asn Leu Pro Asp	Tyr Leu Asp Phe Ala	Glu Ser Gly Gln Val

- 30 -

```

          140                      145                      150
Tyr Phe Gly Ile Ile  Ala Leu
          155

```

5

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 157 amino acids

(B) TYPE: amino acids

15

(C) TOPOLOGY: linear

(ii) MOLECULAR TYPE: protein .

(ix) FEATURE:

20

(A) NAME/KEY: Tumor Necrosis Factor (bovine)

(B) OTHER INFORMATION: A blank residue designated by "Xaa" is inserted after residue No. 70 of bovine TNF and the sequence numbering is augmented by 1 starting with residue No. 71 in order to maximize the sequence homology with human TNF.

25

(x) PUBLICATION INFORMATION:

30

(A) AUTHORS: Niitsu, Y. and Watanabe, N.

(B) TITLE: Cytokines and Receptors - Their Functions, Structures and Cloning of Code Genes. Tumor Necrosis Factor.

35

(C) JOURNAL: Nippon Rinsho

40

(D) VOLUME: 46

(E) PAGES: 1041-1049

(F) DATE: 1988

45

(G) RELEVANT RESIDUES IN SEQ ID NO:3: 1-157 (includes one blank)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

50

Leu Arg Ser Ser Ser Gln Ala Ser Ser Asn Lys Pro Val Ala His
 5 10 15

- 31 -

	Val Val Ala Asp Ile	Asn Ser Pro Gly Gln	Leu Arg Trp Trp Asp
	20	25	30
5	Ser Tyr Ala Asn Ala	Leu Met Ala Asn Gly	Val Gln Leu Glu Asp
	35	40	45
	Asn Gln Leu Val Val	Pro Ala Glu Gly Leu	Tyr Leu Ile Tyr Ser
	50	55	60
10	Gln Val Leu Phe Arg	Gly Gln Gly Cys Pro	Xaa Pro Pro Pro Val
	65	70	75
	Leu Thr His Thr Ile	Ser Arg Ile Ala Val	Ser Tyr Gln Thr Lys
15	80	85	90
	Val Asn Ile Leu Ser	Ala Ile Lys Ser Pro	Cys His Arg Glu Thr
	95	100	105
20	Pro Glu Trp Ala Glu	Ala Lys Pro Trp Tyr	Glu Pro Ile Tyr Gln
	110	115	120
	Gly Gly Val Phe Gln	Leu Glu Lys Asp Asp	Arg Leu Ser Ala Glu
	125	130	135
25	Ile Asn Leu Pro Asp	Tyr Leu Asp Tyr Ala	Glu Ser Gly Gln Val
	140	145	150
	Tyr Phe Gly Ile Ile	Ala Leu	
30	155		

(5) INFORMATION FOR SEQ ID NO:4:

35 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 157 amino acids

40 (B) TYPE: amino acids

(C) TOPOLOGY: linear

(ii) MOLECULAR TYPE: protein

45 (ix) FEATURE:

(A) NAME/KEY: Tumor Necrosis Factor (goat)

50 (B) OTHER INFORMATION: A blank residue designated by "Xaa" is inserted after residue No. 107 of goat TNF and the sequence numbering is augmented by 1 starting with residue No. 108 in order to maximize

- 32 -

the sequence homology with human TNF.

(x) PUBLICATION INFORMATION:

5 (A) JOURNAL: Submitted to EMBL Data Bank X14828

(B) DATE: March 1989

10 (G) RELEVANT RESIDUES IN SEQ ID NO:4: 1-157 (includes one blank)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

15	Leu Arg Ser Ser Ser	Gln Ala Ser Ser Asn	Lys Pro Val Ala His
	5	10	15
	Val Val Ala Asn Ile	Ser Ala Pro Gly Gln	Leu Arg Trp Gly Asp
	20	25	30
20	Ser Tyr Ala Asn Ala	Leu Lys Ala Asn Gly	Val Glu Leu Lys Asp
	35	40	45
	Asn Gln Leu Val Val	Pro Thr Asp Gly Leu	Tyr Leu Ile Tyr Ser
25	50	55	60
	Gln Val Leu Phe Arg	Gly His Gly Cys Pro	Ser Thr Pro Leu Phe
	65	70	75
30	Leu Thr His Thr Ile	Ser Arg Ile Ala Val	Ser Tyr Gln Thr Lys
	80	85	90
	Val Asn Ile Leu Ser	Ala Ile Lys Ser Pro	Cys His Arg Glu Thr
	95	100	105
35	Pro Glu Xaa Ala Glu	Ala Lys Pro Trp Tyr	Glu Pro Ile Tyr Gln
	110	115	120
	Gly Gly Val Phe Gln	Leu Glu Lys Gly Asp	Arg Leu Ser Ala Glu
40	125	130	135
	Ile Asn Gln Pro Glu	Tyr Leu Asp Tyr Ala	Glu Ser Gly Gln Val
	140	145	150
45	Tyr Phe Gly Ile Ile	Ala Leu	
	155		

(6) INFORMATION FOR SEQ ID NO:5:

50 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 157 amino acids

- 33 -

(B) TYPE: amino acids

(C) TOPOLOGY: linear

5 (ii) MOLECULAR TYPE: protein

(ix) FEATURE:

(A) NAME/KEY: Tumor Necrosis Factor (ovine)

10 (x) PUBLICATION INFORMATION:

(A) AUTHORS: Young, A.J., Hay, J.B., and Chan, J.Y.C.

15 (B) TITLE: Primary Structure of Ovine Tumor Necrosis Factor Alpha cDNA.

(C) JOURNAL: Nucleic Acids Research

20 (D) VOLUME: 18

(E) PAGE: 6723

25 (F) DATE: 1990

(G) RELEVANT RESIDUES IN SEQ ID NO:5: 1-157

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

30 Leu Arg Ser Ser Ser Gln Ala Ser Asn Asn Lys Pro Val Ala His
5 10 15

35 Val Val Ala Asn Leu Ser Ala Pro Gly Gln Leu Arg Trp Gly Asp
20 25 30

Ser Tyr Ala Asn Ala Leu Met Ala Asn Gly Val Glu Leu Lys Asp
35 40 45

40 Asn Gln Leu Val Val Pro Thr Asp Gly Leu Tyr Leu Ile Tyr Ser
50 55 60

Gln Val Leu Phe Arg Gly His Gly Cys Pro Ser Thr Pro Leu Phe
45 65 70 75

Leu Thr His Thr Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys
80 85 90

50 Val Asn Ile Leu Ser Ala Ile Lys Ser Pro Cys His Arg Glu Thr
95 100 105

Leu Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Gln

- 34 -

		110		115		120
	Gly Gly Val Phe	Gln	Leu Glu Lys Gly	Asp	Arg Leu Ser Ala	Glu
		125		130		135
5	Ile Asn Leu Pro	Glu	Tyr Leu Asp Tyr	Ala	Glu Ser Gly Gln	Val
		140		145		150
10	Tyr Phe Gly Ile	Ile	Ala Leu			
		155				

(7) INFORMATION FOR SEQ ID NO:6:

15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 157 amino acids

(B) TYPE: amino acids

20 (C) TOPOLOGY: linear

(ii) MOLECULAR TYPE: protein

25 (ix) FEATURE:

(A) NAME/KEY: Tumor Necrosis Factor (feline)

30 (x) PUBLICATION INFORMATION:

(A) AUTHORS: McGraw, R. A., Coffee, B.W., Otto, C.M., Drews, R.T. and Rawling, C.A.

35 (B) TITLE: Gene Sequence of Feline Tumor Necrosis Factor α .

(C) JOURNAL: Nucleic Acids Research

(D) VOLUME: 18

40 (E) PAGE: 5564

(F) DATE: 1990

45 (G) RELEVANT RESIDUES IN SEQ ID NO:6: 1-157

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

50	Leu Arg Ser Ser Ser	Arg Thr Pro Ser Asp	Lys Pro Val Ala His
	5	10	15
	Val Val Ala Asn Pro	Glu Ala Glu Gly Gln	Leu Gln Arg Leu Ser

- 35 -

	20	25	30
5	Arg Arg Ala Asn Ala 35	Leu Leu Ala Asn Gly 40	Val Glu Leu Thr Asp 45
	Asn Gln Leu Lys Val 50	Pro Ser Asp Gly Leu 55	Tyr Leu Ile Tyr Ser 60
10	Gln Val Leu Phe Thr 65	Gly Gln Gly Cys Pro 70	Ser Thr His Val Leu 75
	Leu Thr His Ala Ile 80	Ser Arg Phe Ala Val 85	Ser Tyr Gln Thr Lys 90
15	Val Asn Leu Leu Ser 95	Ala Ile Lys Ser Pro 100	Cys Gln Arg Glu Thr 105
	Pro Glu Gly Ala Glu 110	Ala Lys Pro Trp Tyr 115	Glu Pro Ile Tyr Leu 120
20	Gly Gly Val Phe Gln 125	Leu Glu Lys Gly Asp 130	Arg Leu Ser Thr Glu 135
	Ile Asn Leu Pro Ala 140	Tyr Leu Asp Phe Ala 145	Glu Ser Gly Gln Val 150
25	Tyr Phe Gly Ile Ile 155	Ala Leu	

30 (8) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 157 amino acids

(B) TYPE: amino acids

(C) TOPOLOGY: linear

40 (ii) MOLECULAR TYPE: protein

(ix) FEATURE:

45 (A) NAME/KEY: Tumor Necrosis Factor (rabbit)

(B) OTHER INFORMATION: A blank residue designated by "Xaa" is inserted after residue No. 70 of rabbit TNF and the sequence numbering is augmented by 1 starting with residue No. 71 in order to maximize the sequence homology with human TNF.

(x) PUBLICATION INFORMATION:

- 36 -

- (A) AUTHORS: Ito, H., Shirai, T., Yamamoto, S., Akira, M., Kawahara, S., Todd, C.W. and Wallace, R.B.
- 5 (B) TITLE: Molecular Cloning of the Gene Encoding Rabbit Tumor Necrosis Factor.
- (C) JOURNAL: DNA
- 10 (D) VOLUME: 5
- (E) PAGES: 157-165
- (F) DATE: 1986
- 15 (G) RELEVANT RESIDUES IN SEQ ID NO:7: 1-157 (includes one blank)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

20 Leu Arg Ser Ala Ser Arg Ala Leu Ser Asp Lys Pro Leu Ala His
5 10 15

25 Val Val Ala Asn Pro Gln Val Glu Gly Gln Leu Gln Trp Leu Ser
20 25 30

Gln Arg Ala Asn Ala Leu Leu Ala Asn Gly Met Lys Leu Thr Asp
35 40 45

30 Asn Gln Leu Val Val Pro Ala Asp Gly Leu Tyr Leu Ile Tyr Ser
50 55 60

Gln Val Leu Phe Ser Gly Gln Gly Cys Arg Xaa Ser Tyr Val Leu
65 70 75

35 Leu Thr His Thr Val Ser Arg Phe Ala Val Ser Tyr Pro Asn Lys
80 85 90

40 Val Asn Leu Leu Ser Ala Ile Lys Ser Pro Cys His Arg Glu Thr
95 100 105

Pro Glu Glu Ala Glu Pro Met Ala Trp Tyr Glu Pro Ile Tyr Leu
110 115 120

45 Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Thr Glu
125 130 135

Val Asn Gln Pro Glu Tyr Leu Asp Leu Ala Glu Ser Gly Gln Val
140 145 150

50 Tyr Phe Gly Ile Ile Ala Leu
155

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(9) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 157 amino acids

(B) TYPE: amino acids

(C) TOPOLOGY: linear

(ii) MOLECULAR TYPE: protein

(ix) FEATURE:

15 (A) NAME/KEY: Tumor Necrosis Factor (rat)

20 (B) OTHER INFORMATION: A blank residue designated by "Xaa" is inserted after residue No. 70 of rat TNF and the sequence numbering is augmented by 1 starting with residue No. 71 in order to maximize the sequence homology with human TNF.

(x) PUBLICATION INFORMATION:

25 (A) AUTHORS: Shirai, T., Shimizu, N., Horiguchi,
S., and Ito, H.

30 (B) TITLE: Cloning and Expression in
Escherichia coli of the gene for
Rat

(C) JOURNAL: Agric. Biol. Chem.

(D) VOLUME: 53

(E) PAGES: 1733-1736

(F) DATE: 1989

40 (G) RELEVANT RESIDUES IN SEQ ID NO:8: 1-157 (includes
one blank)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

[illegible]

Val Val Ala Asn His Gln Ala Glu Glu Gln Leu Glu Trp Leu Ser
20 25 30

Gln Arg Ala Asn
Ala Leu Leu Ala Asn Gly Met Asp Leu Lys Asp

35 40 45

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	Asn	Gln	Leu	Val	Val	Pro	Ala	Asp	Gly	Leu	Tyr	Leu	Ile	Tyr	Ser
					50					55					60
5	Gln	Val	Leu	Phe	Lys	Gly	Gln	Gly	Cys	Pro	Xaa	Asp	Tyr	Val	Leu
					65					70					75
	Leu	Thr	His	Thr	Val	Ser	Arg	Phe	Ala	Ile	Ser	Tyr	Gln	Glu	Lys
					80					85					90
10	Val	Ser	Leu	Leu	Ser	Ala	Ile	Lys	Ser	Pro	Cys	Pro	Lys	Asp	Thr
					95					100					105
	Pro	Glu	Gly	Ala	Glu	Leu	Lys	Pro	Trp	Tyr	Glu	Pro	Met	Tyr	Leu
15					110					115					120
	Gly	Gly	Val	Phe	Gln	Leu	Glu	Lys	Gly	Asp	Leu	Leu	Ser	Ala	Glu
					125					130					135
20	Val	Asn	Leu	Pro	Lys	Tyr	Leu	Asp	Ile	Thr	Glu	Ser	Gly	Gln	Val
					140					145					150
	Tyr	Phe	Gly	Val	Ile	Ala	Leu								
					155										

25 (10) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 157 amino acids

(B) TYPE: amino acids

(C) TOPOLOGY: linear

35 (ii) MOLECULAR TYPE: protein

(ix) FEATURE:

40 (A) NAME/KEY: Tumor Necrosis Factor (murine)

(B) OTHER INFORMATION: A blank residue designated by "Xaa" is inserted after residue No. 70 of murine TNF and the sequence numbering is augmented by 1 starting with residue No. 71 in order to maximize the sequence homology with human TNF.

(x) PUBLICATION INFORMATION:

50 (A) AUTHORS: Caput, D., Beutler, B. Hartog, K. Thayer, R., Brown-Shimer, S. and Cerami, A.

- 39 -

- (B) TITLE: Identification of a Common Nucleotide Sequence in the 3'-Untranslated Region of mRNA Molecules Specifying Inflammatory Mediators.
- 5 (C) JOURNAL: Proc. National Academy of Science, U.S.A.
- (D) VOLUME: 83
- 10 (E) PAGES: 1670-1674
- (F) DATE: 1986
- 15 (G) RELEVANT RESIDUES IN SEQ ID NO:9: 1-157 (includes one blank)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9: murine TNF

20	Leu Arg Ser Ser Ser	Gln Asn Ser Ser Asp	Lys Pro Val Ala His
	5	10	15
	Val Val Ala Asn His	Gln Val Glu Glu Gln	Leu Glu Trp Leu Ser
	20	25	30
25	Gln Arg Ala Asn Ala	Leu Leu Ala Asn Gly	Met Asp Leu Lys Asp
	35	40	45
	Asn Gln Leu Val Val	Pro Ala Asp Gly Leu	Tyr Leu Val Tyr Ser
30	50	55	60
	Gln Val Leu Phe Lys	Gly Gln Gly Cys Pro	Xaa Asp Val Val Leu
	65	70	75
35	Leu Thr His Thr Val	Ser Arg Phe Ala Ile	Ser Tyr Gln Glu Lys
	80	85	90
	Val Asn Leu Leu Ser	Ala Val Lys Ser Pro	Cys Pro Lys Asp Thr
	95	100	105
40	Pro Glu Gly Ala Glu	Leu Lys Pro Trp Tyr	Glu Pro Ile Tyr Leu
	110	115	120
	Gly Gly Val Phe Gln	Leu Glu Lys Gly Asp	Gln Leu Ser Ala Glu
45	125	130	135
	Val Asn Leu Pro Lys	Tyr Leu Asp Phe Ala	Glu Ser Gly Gln Val
	140	145	150
50	Tyr Phe Gly Val Ile	Ala Leu	
	155		

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(11) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 171 amino acids

(B) TYPE: amino acids

10 (C) TOPOLOGY: linear

(ii) MOLECULAR TYPE: protein

(ix) FEATURE:

15 (A) NAME/KEY: Lymphotoxin (human)

(x) PUBLICATION INFORMATION:

20 (A) AUTHORS: Hedwin, G.E., Naylor, S.L.,
Sakaguchi, A.Y., Smith, D., Nedwin,
J.J., Pennica, D., Goeddel, D.V.,
et al.25 (B) TITLE: Human Lymphotoxin and Tumor Necrosis
Factor: Structure, Homology and
Chromosomal Localization.

30 (C) JOURNAL: Nucleic Acids Research

(D) VOLUME: 13

(E) PAGES: 6261-6373

35 (F) DATE: 1985

(G) RELEVANT RESIDUES IN SEQ ID NO:10: 1-171

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10 human lt

40	Leu Pro Gly Val Gly	Leu Thr Pro Ser Ala	Ala Gln Thr Ala Arg
	5	10	15
	Gln His Pro Lys Met	His Leu Ala His Ser	Thr Leu Lys Pro Ala
45	20	25	30
	Ala His Leu Ile Gly	Asp Pro Ser Lys Gln	Asn Ser Leu Leu Trp
	35	40	45
50	Arg Ala Asn Thr Asp	Arg Ala Phe Leu Gln	Asp Gly Phe Ser Leu
	50	55	60

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	Ser	Asn	Asn	Ser	Leu	Leu	Val	Pro	Thr	Ser	Gly	Ile	Tyr	Phe	Val
					65					70					75
5	Tyr	Ser	Gln	Val	Val	Phe	Ser	Gly	Lys	Ala	Tyr	Ser	Pro	Lys	Ala
					80					85					90
	Thr	Ser	Ser	Pro	Leu	Tyr	Leu	Ala	His	Glu	Val	Gln	Leu	Phe	Ser
					95					100					105
10	Ser	Gln	Tyr	Pro	Phe	His	Val	Pro	Leu	Leu	Ser	Ser	Gln	Lys	Met
					110					115					120
	Val	Tyr	Pro	Gly	Leu	Gln	Glu	Pro	Trp	Leu	His	Ser	Met	Tyr	His
15					125					130					135
	Gly	Ala	Ala	Phe	Gln	Leu	Thr	Gln	Gly	Asp	Gln	Leu	Ser	Thr	His
					140					145					150
20	Thr	Asp	Gly	Ile	Pro	His	Leu	Val	Leu	Ser	Pro	Ser	Thr	Val	Phe
					155					160					165
	Phe	Gly	Ala	Phe	Ala	Leu									
					170										

25

(12) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

30

(A) LENGTH: 171 amino acids

(B) TYPE: amino acids

(C) TOPOLOGY: linear

35

(ii) MOLECULAR TYPE: protein

(ix) FEATURE:

40

(A) NAME/KEY: Lymphotoxin (bovine)

(x) PUBLICATION INFORMATION:

45

(A) AUTHORS: Niitsu, Y. and Watanabe, N.

(B) TITLE: Cytokines and Receptors - Their Functions, Structures and Cloning of Code Genes. Tumor Necrosis Factor.

50

(C) JOURNAL: Nippon Rinsho

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(D) VOLUME: 46

(E) PAGES: 1041-1049

5 (F) DATE: 1988

(G) RELEVANT RESIDUES IN SEQ ID NO:11: 1-171

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11: bovine lt

10	Leu Arg Gly Ile Gly	Leu Thr Pro Ser Ala	Ala Gln Pro Ala His
	5	10	15
15	Gln Gln Leu Pro Thr	Pro Phe Thr Arg Gly	Thr Leu Lys Pro Ala
	20	25	30
	Ala His Leu Val Gly	Asp Pro Ser Thr Gln	Asp Ser Leu Arg Trp
	35	40	45
20	Arg Ala Asn Thr Asp	Arg Ala Phe Leu Arg	His Gly Phe Ser Leu
	50	55	60
25	Ser Asn Asn Ser Leu	Leu Val Pro Thr Ser	Gly Leu Tyr Phe Val
	65	70	75
	Tyr Ser Gln Val Val	Phe Ser Gly Arg Gly	Cys Phe Pro Arg Ala
	80	85	90
30	Thr Pro Thr Pro Leu	Tyr Leu Ala His Glu	Val Gln Leu Phe Ser
	95	100	105
	Pro Gln Tyr Pro Phe	His Val Pro Leu Leu	Ser Ala Gln Lys Ser
	110	115	120
35	Val Cys Pro Gly Pro	Gln Gly Pro Trp Val	Arg Ser Val Tyr Gln
	125	130	135
40	Gly Ala Val Phe Leu	Leu Thr Arg Gly Asp	Gln Leu Ser Thr His
	140	145	150
	Thr Asp Gly Ile Ser	His Leu Leu Leu Ser	Pro Ser Ser Val Phe
	155	160	165
45	Phe Gly Ala Phe Ala	Leu	
	170		

(13) INFORMATION FOR SEQ ID NO:12:

50 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 171 amino acids

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(B) TYPE: amino acids

(C) TOPOLOGY: linear

5 (ii) MOLECULAR TYPE: protein

(ix) FEATURE:

10 (A) NAME/KEY: Lymphotoxin (rabbit)

(B) OTHER INFORMATION: Two blank residues designated by "Xaa" are inserted after residue No. 34 and No. 61 of murine lymphotoxin and the sequence numbering is augmented by 1 starting with residue No. 35 and again augmented by 1 starting with residue No. 62 in order to maximize the sequence homology with human lymphotoxin.

15

20 (x) PUBLICATION INFORMATION:

(A) AUTHORS: Ito, H., Shirai, T., Yamamoto, S., Akira, M., Kawahara, S., Todd, C.W. and Wallace, R.B.

25 (B) TITLE: Molecular Cloning of the Gene Encoding Rabbit Tumor Necrosis Factor.

(C) JOURNAL: DNA

30 (D) VOLUME: 5

(E) PAGES: 157-165

35 (F) DATE: 1986

(G) RELEVANT RESIDUES IN SEQ ID NO:12: 1-171 (includes 2 blanks)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

40 Leu Pro Gly Ala Gln Phe Pro Pro Ser Ala Ala Arg Asn Ala Gln
5 10 15

45 Gln Arg Leu Gln Lys His Phe Gly His Ser Thr Leu Lys Pro Ala
20 25 30

Ala His Leu Val Xaa Asp Pro Ser Ala Gln Asp Ser Leu Arg Trp
35 40 45

50 Arg Ala Asn Thr Asp Arg Ala Phe Leu Ala His Gly Phe Ser Leu
50 55 60

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	Ser	Asn	Xaa	Phe	Pro	Cys	Gly	Pro	Ser	Ser	Gly	Leu	Tyr	Phe	Val
					65					70					75
5	Tyr	Ser	Gln	Val	Val	Phe	Ser	Gly	Glu	Gly	Cys	Ser	Pro	Lys	Ala
					80					85					90
	Val	Pro	Thr	Pro	Leu	Tyr	Leu	Ala	His	Glu	Val	His	Leu	Phe	Ser
					95					100					105
10	Ser	Gln	Tyr	Ser	Phe	His	Val	Pro	Leu	Leu	Ser	Ala	Gln	Lys	Ser
					110					115					120
	Val	Cys	Pro	Gly	Pro	Gln	Gly	Pro	Trp	Val	Arg	Ser	Val	Tyr	Gln
					125					130					135
15	Gly	Ala	Val	Phe	Leu	Leu	Thr	Gln	Gly	Glu	Gln	Leu	Ser	Thr	His
					140					145					150
	Thr	Asp	Gly	Ile	Ala	His	Leu	Leu	Leu	Ser	Pro	Ser	Ser	Val	Phe
20					155					160					165
	Phe	Gly	Ala	Phe	Ala	Leu									
					170										

25 (14) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 171 amino acids

(B) TYPE: amino acids

(C) TOPOLOGY: linear

35 (ii) MOLECULAR TYPE: protein

(ix) FEATURE:

40 (A) NAME/KEY: Lymphotoxin (murine)

(B) OTHER INFORMATION: Two blank residues designated by "Xaa" are inserted after residue No. 4 of murine lymphotoxin and the sequence numbering is augmented by 2 starting with residue No. 5 in order to maximize the sequence homology with human lymphotoxin.

(x) PUBLICATION INFORMATION:

50 (A) AUTHORS: Li, C-B., Gray, R.W., Lin, P-F., McGrath, K.M. and Ruddle, F.H., Ruddle, N.H.

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(B) TITLE: Cloning and Expression of Murine Lymphotoxin cDNA.

(C) JOURNAL: J. Immunology

(D) VOLUME: 138

(E) PAGES: 4496-4501

(F) DATE: 1987

(G) RELEVANT RESIDUES IN SEQ ID NO:13: 1-171 (includes two blanks)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Leu Ser Gly Val Xaa	Xaa Arg Phe Ser Ala	Ala Arg Thr Ala His
5	10	15
Pro Leu Pro Gln Lys	His Leu Thr His Gly	Ile Leu Lys Pro Ala
20	25	30
Ala His Leu Val Gly	Tyr Pro Ser Lys Gln	Asn Ser Leu Leu Trp
35	40	45
Arg Ala Ser Thr Asp	Arg Ala Phe Leu Arg	His Gly Phe Ser Leu
50	55	60
Ser Asn Asn Ser Leu	Leu Ile Pro Thr Ser	Gly Leu Tyr Phe Val
65	70	75
Tyr Ser Gln Val Val	Phe Ser Gly Glu Ser	Cys Ser Pro Arg Ala
80	85	90
Ile Pro Thr Pro Ile	Tyr Leu Ala His Glu	Val Gln Leu Phe Ser
95	100	105
Ser Gln Tyr Pro Phe	His Val Pro Leu Leu	Ser Ala Gln Lys Ser
110	115	120
Val Tyr Pro Gly Leu	Gln Gly Pro Trp Val	Arg Ser Met Tyr Gln
125	130	135
Gly Ala Val Phe Leu	Leu Ser Lys Gly Asp	Gln Leu Ser Thr His
140	145	150
Thr Asp Gly Ile Ser	His Leu His Phe Ser	Pro Ser Ser Val Phe
155	160	165
Phe Gly Ala Phe Ala	Leu	
170		

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What is claimed is:

1. A method for making a modified form of TNF- α having a reconstructed channel, as compared to unmodified TNF- α , for regulating channel activity, the method comprising the following steps:

Step A: forming one or more candidate forms of modified TNF- α by substituting one or more channel residues with replacement amino acids, the channel residues being selected from the group consisting of the following sequence numbers:

residue #11; residue #57; residue # 59;
residue #98; residue #112; residue #
116; residue #119; residue #121;
residue #155; and residue #157; the
sequence numbers being defined with
respect to unmodified forms of human
TNF- α ,

the replacement amino acid for residue #11
being selected from the group
consisting of: Glu, Arg, Cys, Asp, Gln,
Asn, Ser, Thr, and His;

the replacement amino acid for residue #57
being selected from the group
consisting of: Trp, Ser, Thr, Ala, Met,
Cys, Phe, and Tyr;

the replacement amino acid for residue #59
being selected from the group
consisting of: Trp, Ser, Thr, Ala, Met,
Cys, and Phe;

the replacement amino acid for residue #98
being selected from the group
consisting of: Arg, Cys, Glu, Asp, Gln,
Asn, Ser, Thr, and His;

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- the replacement amino acid for residue #112
being selected from the group
consisting of: Arg, Cys, Asp, Gln, Asn,
Ser, Thr, Glu and His;
- 5 the replacement amino acid for residue #116
being selected from the group
consisting of: Lys, Arg, Cys, Asp, Gln,
Asn, Ser, His, and Thr;
- 10 the replacement amino acid for residue #119
being selected from the group
consisting of: Trp, Phe, Ser, Thr, Ala,
Met, and Cys;
- the replacement amino acid for residue #121
being selected from the group
15 consisting of: Ala, Val, Ser, and Thr;
- the replacement amino acid for residue #155
being selected from the group
consisting of: Trp, Ser, Thr, Ala, Met,
Cys, Phe, and Tyr; and
- 20 the replacement amino acid for residue #157
being selected from the group
consisting of: Trp, Ser, Thr, Ala,
Cys, and Tyr;
- Step B: determining whether the candidate form of
25 TNF- α has an ability to form TNF trimers;
- Step C: determining whether the candidate form of
TNF- α has an ability to achieve intimate contact
with a target that includes both a membrane and
one or more TNF receptors;
- 30 Step D: determining whether the candidate form of
TNF- α , when in intimate contact with the target,
achieves a modified effect, the modified effect
being of a type caused by a modified channel
activity of the candidate form of TNF- α , the
35 modified channel activity materially differing

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from corresponding unmodified channel activities of unmodified TNF- α ; then

5 Step E: selecting the modified form of TNF- α from one or more of the candidate forms of TNF- α , the modified form of TNF- α having been determined in said Step B to be able to form TNF trimers, in said Step C to be able to achieve intimate contact with the target, and in said Step D to be able to achieve the modified effect by virtue of the modified channel activity; and then

10 Step F: making the modified form of TNF- α selected in said Step E in purified form and in commercial quantities.

15 2. A method for making a modified form of TNF- α as described in claim 1 wherein:

in said Step A:

the replacement amino acid for residue #11 being further selected from the group consisting of: Val, Leu, Ile, and Ala;

20 the replacement amino acid for residue #57 being further selected from the group consisting of: Arg, Glu, Lys, Asp, Gln, and Asn;

25 the replacement amino acid for residue #59 being further selected from the group consisting of: Arg, Glu, Lys, Asp, Gln, and Asn;

30 the replacement amino acid for residue #98 being further selected from the group consisting of: Val, Leu, Ile, and Ala;

the replacement amino acid for residue #112 being further selected from the group consisting of: Val, Leu, Ile, and Ala;

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- the replacement amino acid for residue #116
being further selected from the group
consisting of: Leu, Ile, and Ala;
- 5 the replacement amino acid for residue #119
being further selected from the group
consisting of: Arg, Glu, Lys, Asp, Gln,
and Asn;
- 10 the replacement amino acid for residue #121
being further selected from the group
consisting of: Pro, Ile, Leu, and His;
- the replacement amino acid for residue #155
being further selected from the group
consisting of: Arg, Glu, Lys, Asp,
Gln, and Asn; and
- 15 the replacement amino acid for residue #157
being further selected from the group
consisting of: Arg, Glu, Lys, Asp, and
Asn.
- 20 3. A method for making a modified form of TNF- α as
described in claim 2 wherein:
in said Step A:
- 25 the replacement amino acid for residue #11
being further selected from the group
consisting of: Trp, Gly, Pro, Tyr, Phe,
and Met;
- the replacement amino acid for residue #57
being further selected from the group
consisting of: Gly, Val, Ile, His, and
30 Pro;
- the replacement amino acid for residue #59
being further selected from the group
consisting of: Gly, Val, Leu, Ile,
His, and Pro;
- 35 the replacement amino acid for residue #98
being further selected from the group

- consisting of: Trp, Met, Gly, Pro, Tyr,
and Phe;
the replacement amino acid for residue #112
being further selected from the group
5 consisting of: Trp, Gly, Pro, Tyr, and
Phe;
the replacement amino acid for residue #116
being further selected from the group
consisting of: Trp, Met, Gly, Pro, Tyr,
10 and Phe;
the replacement amino acid for residue #119
being further selected from the group
consisting of: Gly, Val, Ile, Leu, and
Pro;
15 the replacement amino acid for residue #121
being further selected from the group
consisting of: Trp, Tyr, Phe, Cys, Met,
Lys, Glu, Arg, Gln, Asp, and Asn;
the replacement amino acid for residue #155
20 being further selected from the group
consisting of: Gly, Val, His, and Pro;
and
the replacement amino acid for residue #157
being further selected from the group
25 consisting of: Gly, Ile, and His.
4. A method for making a modified form of TNF- α as
described in claim 3 wherein:
- 30 in said Step D: the modified effect achieved by the
modified TNF- α with the target being of a type
caused by a modified channel activity that is
materially reduced as compared to the
corresponding unmodified channel activity of
unmodified TNF- α ; and
35 in said Step E: selecting as the modified form of
TNF- α a candidate form of TNF- α that is further

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determined to achieve the modified effect by virtue of the modified channel activity that is materially reduced.

- 5 5. A method for making a modified form of TNF- α as described in claim 3 wherein:

10 in said Step D: the modified effect achieved by the modified TNF- α with the target being of a type caused by a modified channel activity that is materially enhanced as compared to the corresponding unmodified channel activity of unmodified TNF- α ; and

15 in said Step E: selecting as the modified form of TNF- α a candidate form of TNF- α that is further determined to achieve the modified effect by virtue of the modified channel activity that is materially enhanced.

- 20 6. A modified form of TNF- α having a reconstructed channel, as compared to unmodified TNF- α , for regulating channel activity, the modified form of TNF- α being constructed by the following steps:

25 Step A: forming one or more candidate forms of modified TNF- α by substituting one or more channel residues with replacement amino acids, the channel residues being selected from the group consisting of the following sequence numbers:

30 residue #11; residue #57; residue # 59;
residue #98; residue #112; residue #
116; residue #119; residue #121;
residue #155; and residue #157; the
sequence numbers being defined with
35 respect to unmodified forms of human
TNF- α ,

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- the replacement amino acid for residue #11
being selected from the group
consisting of: Glu, Arg, Cys, Asp,
Gln, Asn, Ser, Thr, and His;
- 5 the replacement amino acid for residue #57
being selected from the group
consisting of: Trp, Ser, Thr, Ala, Met,
Cys, Phe, and Tyr;
- 10 the replacement amino acid for residue #59
being selected from the group
consisting of: Trp, Ser, Thr, Ala, Met,
Cys, and Phe;
- 15 the replacement amino acid for residue #98
being selected from the group
consisting of: Arg, Cys, Glu, Asp, Gln,
Asn, Ser, Thr, and His;
- 20 the replacement amino acid for residue #112
being selected from the group
consisting of: Arg, Cys, Asp, Gln, Asn,
Ser, Thr, Glu, and His;
- 25 the replacement amino acid for residue #116
being selected from the group
consisting of: Lys, Arg, Cys, Asp, Gln,
Asn, Ser, His, and Thr;
- 30 the replacement amino acid for residue #119
being selected from the group
consisting of: Trp, Phe, Ser, Thr, Ala,
Met, and Cys;
- 35 the replacement amino acid for residue #121
being selected from the group
consisting of: Ala, Val, Ser, and Thr;
- the replacement amino acid for residue #155
being selected from the group
consisting of: Trp, Ser, Thr, Ala, Met,
Cys, Phe, and Tyr; and

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the replacement amino acid for residue #157
being selected from the group
consisting of: Trp, Ser, Thr, Ala,
Cys, and Tyr;

5 Step B: determining whether the candidate form of
TNF- α has an ability to form TNF trimers;

Step C: determining whether the candidate form of
TNF- α has an ability to achieve intimate contact
with a target that includes both a membrane and
10 one or more TNF receptors;

Step D: determining whether the candidate form of
TNF- α , when in intimate contact with the target,
achieves a modified effect, the modified effect
being of a type caused by a modified channel
15 activity of the candidate form of TNF- α , the
modified channel activity materially differing
from corresponding unmodified channel activities
of unmodified TNF- α ; then

Step E: selecting the modified form of TNF- α from
20 one or more of the candidate forms of TNF- α , the
modified form of TNF- α having been determined in
said Step B to be able to form TNF trimers, in
said Step C to be able to achieve intimate
contact with the target, and in said Step D to be
25 able to achieve the modified effect by virtue of
the modified channel activity; and then

Step F: making the modified form of TNF- α selected
in said Step E in purified form and in commercial
quantities.

30

7. A modified form of TNF- α as described in claim 6
wherein:

in said Step A:

35 the replacement amino acid for residue #11
being further selected from the group
consisting of: Val, Leu, Ile, and Ala;

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- the replacement amino acid for residue #57
being further selected from the group
consisting of: Arg, Glu, Lys, Asp,
Gln, and Asn;
- 5 the replacement amino acid for residue #59
being further selected from the group
consisting of: Arg, Glu, Lys, Asp, Gln,
and Asn;
- 10 the replacement amino acid for residue #98
being further selected from the group
consisting of: Val, Leu, Ile, and Ala;
- the replacement amino acid for residue #112
being further selected from the group
consisting of: Val, Leu, Ile, and Ala;
- 15 the replacement amino acid for residue #116
being further selected from the group
consisting of: Leu, Ile, and Ala;
- the replacement amino acid for residue #119
being further selected from the group
20 consisting of: Arg, Glu, Lys, Asp, Gln,
and Asn;
- the replacement amino acid for residue #121
being further selected from the group
consisting of: Pro, Ile, Leu, and His;
- 25 the replacement amino acid for residue #155
being further selected from the group
consisting of: Arg, Glu, Lys, Asp,
Gln, and Asn; and
- the replacement amino acid for residue #157
30 being further selected from the group
consisting of: Arg, Glu, Lys, Asp, and
Asn.

8. A modified form of TNF- α as described in claim 7
35 wherein:
in said Step A:

- the replacement amino acid for residue #11
being further selected from the group
consisting of: Trp, Gly, Pro, Tyr, Phe,
and Met;
- 5 the replacement amino acid for residue #57
being further selected from the group
consisting of: Gly, Val, Ile, His, and
Pro;
- 10 the replacement amino acid for residue #59
being further selected from the group
consisting of: Gly, Val, Leu, Ile,
His, and Pro;
- 15 the replacement amino acid for residue #98
being further selected from the group
consisting of: Trp, Met, Gly, Pro, Tyr,
and Phe;
- 20 the replacement amino acid for residue #112
being further selected from the group
consisting of: Trp, Gly, Pro, Tyr, and
Phe;
- the replacement amino acid for residue #116
being further selected from the group
consisting of: Trp, Met, Gly, Pro, Tyr,
and Phe;
- 25 the replacement amino acid for residue #119
being further selected from the group
consisting of: Gly, Val, Ile, Leu, and
Pro;
- 30 the replacement amino acid for residue #121
being further selected from the group
consisting of: Trp, Tyr, Phe, Cys, Met,
Lys, Glu, Arg, Gln, Asp, and Asn;
- 35 the replacement amino acid for residue #155
being further selected from the group
consisting of: Gly, Val, His, and Pro;
and

the replacement amino acid for residue #157
being further selected from the group
consisting of: Gly, Ile, and His.

- 5 9. A modified form of TNF- α as described in claim 8
wherein:

in said Step E: determining whether the candidate
form of TNF- α has a channel activity that is
materially reduced as compared to the
10 corresponding channel activity of unmodified TNF-
 α and that is employable for reducing the channel
activity of the target membrane; and
in said Step F: designating as the modified form of
TNF- α a candidate form of TNF- α that is
15 determined to have a channel activity that is
materially reduced with respect to the
corresponding channel activity of unmodified TNF-
 α .

- 20 10. A modified form of TNF- α as described in claim 8
wherein:

in said Step E: determining whether the candidate
form of TNF- α has a channel activity that is
materially enhanced as compared to the
25 corresponding channel activity of unmodified TNF-
 α and that is employable for enhancing the
channel activity of the target membrane; and
in said Step F: designating as the modified form of
TNF- α a candidate form of TNF- α that is
30 determined to have a channel activity that is
materially enhanced with respect to the
corresponding channel activity of unmodified TNF-
 α .

- 35 11. A modified form of TNF- α comprising:

a trimer of three monomers of modified TNF- α , two or more of the three monomers of modified TNF- α within said trimer being covalently bonded to one another by means of disulfide bonds.

5

12. A modified form of TNF- α as described in claim 11 wherein:

the disulfide bond being formed under non-reducing conditions by cysteine molecules introduced by substitution into the modified TNF- α molecule, the cysteine substitutions being introduced into residues selected from the group consisting of the following sequence numbers:

the pair of residues #98 and #116; the pair of residues #103 and #104; the pair of residues #11 and #157; and the single residue #119; the sequence numbers being defined with respect to unmodified forms of human TNF- α .

20

13. A method for making a modified form of TNF- β having a reconstructed channel, as compared to unmodified TNF- β , for regulating channel activity, the method comprising the following steps:

Step A: forming one or more candidate forms of modified TNF- β by substituting one or more channel residues with replacement amino acids, the channel residues being selected from the group consisting of the following sequence numbers:

30

residue #28; residue #74; residue # 76; residue #119; residue #127; residue # 131; residue #134; residue #136; residue #169; and residue #171; the sequence numbers being defined with

35

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respect to unmodified forms of human
TNF- β ,

the replacement amino acid for residue #28
being selected from the group
5 consisting of: Glu, Arg, Cys, Asp, Gln,
Asn, Ser, Thr, and His;

the replacement amino acid for residue #74
being selected from the group
consisting of: Trp, Ser, Thr, Ala, Met,
10 Cys, Leu, and Tyr;

the replacement amino acid for residue #76
being selected from the group
consisting of: Trp, Ser, Thr, Ala, Met,
Cys, and Phe;

15 the replacement amino acid for residue #119
being selected from the group
consisting of: Arg, Cys, Glu, Asp, Gln,
Asn, Ser, Thr, and His;

the replacement amino acid for residue #127
20 being selected from the group
consisting of: Arg, Cys, Asp, Gln, Asn,
Ser, Thr, Lys, and His;

the replacement amino acid for residue #131
being selected from the group
25 consisting of: Lys, Arg, Cys, Asp,
Gln, Asn, Ser, Glu, and Thr;

the replacement amino acid for residue #134
being selected from the group
consisting of: Trp, Phe, Ser, Thr, Ala,
30 Met, and Cys;

the replacement amino acid for residue #136
being selected from the group
consisting of: Ala, Val, Ser, and Thr;

the replacement amino acid for residue #169
35 being selected from the group

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- consisting of: Trp, Ser, Thr, Ala, Met,
Cys, Ile, and Tyr; and
the replacement amino acid for residue #171
being selected from the group
5 consisting of: Trp, Ser, Thr, Ala, Cys,
Phe, and Tyr;
- Step B: determining whether the candidate form of
TNF- β has an ability to form TNF trimers;
- Step C: determining whether the candidate form of
10 TNF- β has an ability to achieve intimate contact
with a target that includes both a membrane and
one or more TNF receptors;
- Step D: determining whether the candidate form of
TNF- β , when in intimate contact with the target,
15 achieves a modified effect, the modified effect
being of a type caused by a modified channel
activity of the candidate form of TNF- β , the
modified channel activity materially differing
from corresponding unmodified channel activities
20 of unmodified TNF- β ; then
- Step E: selecting the modified form of TNF- β from
one or more of the candidate forms of TNF- β , the
modified form of TNF- β having been determined in
said Step B to be able to form TNF trimers, in
25 said Step C to be able to achieve intimate
contact with the target, and in said Step D to be
able to achieve the modified effect by virtue of
the modified channel activity; and then
- Step F: making the modified form of TNF- β selected
30 in said Step E in purified form and in commercial
quantities.
14. A method for making a modified form of TNF- β as
described in claim 13 wherein:
35 in said Step A:

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- the replacement amino acid for residue #28
being further selected from the group
consisting of: Val, Leu, Ile, and Ala;
- 5 the replacement amino acid for residue #74
being further selected from the group
consisting of: Arg, Glu, Lys, Asp, Gln,
and Asn;
- 10 the replacement amino acid for residue #76
being further selected from the group
consisting of: Arg, Glu, Lys, Asp, Gln,
and Asn;
- 15 the replacement amino acid for residue #119
being further selected from the group
consisting of: Val, Leu, Ile, and Ala;
- the replacement amino acid for residue #127
being further selected from the group
consisting of: Val, Leu, Ile, Met, and
Ala;
- 20 the replacement amino acid for residue #131
being further selected from the group
consisting of: Leu, Ile, Val, and Ala;
- the replacement amino acid for residue #134
being further selected from the group
consisting of: Arg, Glu, Lys, Asp, Gln,
25 His, and Asn;
- the replacement amino acid for residue #136
being further selected from the group
consisting of: Pro, Ile, Leu, and His;
- 30 the replacement amino acid for residue #169
being further selected from the group
consisting of: Arg, Glu, Lys, Asp, Gln,
Leu, and Asn; and
- 35 the replacement amino acid for residue #171
being further selected from the group
consisting of: Arg, Glu, Lys, Asp, Gln,
and Asn.

15. A method for making a modified form of TNF- β as described in claim 14 wherein:

in said Step A:

- 5 the replacement amino acid for residue #28
 being further selected from the group
 consisting of: Trp, Gly, Pro, Tyr, Phe,
 and Met;
- 10 the replacement amino acid for residue #74
 being further selected from the group
 consisting of: Gly, Val, Ile, His, and
 Pro;
- 15 the replacement amino acid for residue #76
 being further selected from the group
 consisting of: Gly, Val, Ile, Leu, His,
 and Pro;
- 20 the replacement amino acid for residue #119
 being further selected from the group
 consisting of: Trp, Met, Gly, Pro, Tyr,
 and Phe;
- 25 the replacement amino acid for residue #127
 being further selected from the group
 consisting of: Trp, Gly, Pro, Tyr, and
 Phe;
- 30 the replacement amino acid for residue #131
 being further selected from the group
 consisting of: Trp, Met, Gly, Pro, Tyr,
 and Phe;
- 35 the replacement amino acid for residue #134
 being further selected from the group
 consisting of: Gly, Val, Ile, Leu, and
 Pro;
- the replacement amino acid for residue #136
 being further selected from the group
 consisting of: Trp, Tyr, Phe, Cys, Met,
 Lys, Glu, Arg, Gln, Asp, and Asn;

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the replacement amino acid for residue #169
being further selected from the group
consisting of: Gly, Val, His, and Pro;
and

5 the replacement amino acid for residue #171
being further selected from the group
consisting of: Gly, Pro, Val, Met, Ile,
and His.

10 16. A method for making a modified form of TNF- β as
described in claim 15 wherein:

in said Step D: the modified effect achieved by the
modified TNF- β with the target being of a type
caused by a modified channel activity that is
15 materially reduced as compared to the
corresponding unmodified channel activity of
unmodified TNF- β ; and

in said Step E: selecting as the modified form of
TNF- β a candidate form of TNF- β that is further
20 determined to achieve the modified effect by
virtue of the modified channel activity that is
materially reduced.

17. A method for making a modified form of TNF- β as
25 described in claim 15 wherein:

in said Step D: the modified effect achieved by the
modified TNF- β with the target being of a type
caused by a modified channel activity that is
materially enhanced as compared to the
30 corresponding unmodified channel activity of
unmodified TNF- β ; and

in said Step E: selecting as the modified form of
TNF- β a candidate form of TNF- β that is further
determined to achieve the modified effect by
35 virtue of the modified channel activity that is
materially enhanced.

18. A modified form of TNF- β having a reconstructed channel, as compared to unmodified TNF- β , for regulating channel activity, the modified form of TNF- β being constructed by the following steps:

- 5 Step A: forming one or more candidate forms of modified TNF- β by substituting one or more channel residues with replacement amino acids, the channel residues being selected from the group consisting of the following sequence
- 10 numbers:
- residue #28; residue #74; residue # 76;
 residue #119; residue #127; residue #
 131; residue #134; residue #136;
 residue #169; and residue #171; the
- 15 sequence numbers being defined with respect to unmodified forms of human TNF- β ,
- the replacement amino acid for residue #28 being selected from the group
- 20 consisting of: Glu, Arg, Cys, Asp, Gln, Asn, Ser, Thr, and His;
- the replacement amino acid for residue #74 being selected from the group
- consisting of: Trp, Ser, Thr, Ala, Met,
- 25 Cys, Leu, and Tyr;
- the replacement amino acid for residue #76 being selected from the group
- consisting of: Trp, Ser, Thr, Ala, Met, Cys, and Phe;
- 30 the replacement amino acid for residue #119 being selected from the group
- consisting of: Arg, Cys, Glu, Asp, Gln, Asn, Ser, Thr, and His;
- the replacement amino acid for residue #127
- 35 being selected from the group

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consisting of: Arg, Cys, Asp, Gln, Asn,
Ser, Thr, Lys, and His;
the replacement amino acid for residue #131
being selected from the group
5 consisting of: Lys, Arg, Cys, Asp,
Gln, Asn, Ser, Glu, and Thr;
the replacement amino acid for residue #134
being selected from the group
consisting of: Trp, Phe, Ser, Thr, Ala,
10 Met, and Cys;
the replacement amino acid for residue #136
being selected from the group
consisting of: Ala, Val, Ser, and Thr;
the replacement amino acid for residue #169
15 being selected from the group
consisting of: Trp, Ser, Thr, Ala, Met,
Cys, Ile, and Tyr; and
the replacement amino acid for residue #171
being selected from the group
20 consisting of: Trp, Ser, Thr, Ala, Cys,
Phe, and Tyr;
Step B: determining whether the candidate form of
TNF- β has an ability to form TNF trimers;
Step C: determining whether the candidate form of
25 TNF- β has an ability to achieve intimate contact
with a target that includes both a membrane and
one or more TNF receptors;
Step D: determining whether the candidate form of
TNF- β , when in intimate contact with the target,
30 achieves a modified effect, the modified effect
being of a type caused by a modified channel
activity of the candidate form of TNF- β , the
modified channel activity materially differing
from corresponding unmodified channel activities
35 of unmodified TNF- β ; then

Step E: selecting the modified form of TNF- β from one or more of the candidate forms of TNF- β , the modified form of TNF- β having been determined in said Step B to be able to form TNF trimers, in
5 said Step C to be able to achieve intimate contact with the target, and in said Step D to be able to achieve the modified effect by virtue of the modified channel activity; and then
Step F: making the modified form of TNF- β selected
10 in said Step E in purified form and in commercial quantities.

19. A modified form of TNF- β as described in claim 18 wherein:

15 in said Step A:
the replacement amino acid for residue #28 being further selected from the group consisting of: Val, Leu, Ile, and Ala;
the replacement amino acid for residue #74
20 being further selected from the group consisting of: Arg, Glu, Lys, Asp, Gln, and Asn;
the replacement amino acid for residue #76 being further selected from the group
25 consisting of: Arg, Glu, Lys, Asp, Gln, and Asn;
the replacement amino acid for residue #119 being further selected from the group consisting of: Val, Leu, Ile, and Ala;
30 the replacement amino acid for residue #127 being further selected from the group consisting of: Val, Leu, Ile, Met, and Ala;
the replacement amino acid for residue #131
35 being further selected from the group consisting of: Leu, Ile, Val, and Ala;

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- the replacement amino acid for residue #134
being further selected from the group
consisting of: Arg, Glu, Lys, Asp, Gln,
His, and Asn;
- 5 the replacement amino acid for residue #136
being further selected from the group
consisting of: Pro, Ile, Leu, and His;
- the replacement amino acid for residue #169
being further selected from the group
10 consisting of: Arg, Glu, Lys, Asp, Gln,
Leu, and Asn; and
- the replacement amino acid for residue #171
being further selected from the group
consisting of: Arg, Glu, Lys, Asp, Gln,
15 and Asn.
20. A modified form of TNF- β as described in claim 19
wherein:
- in said Step A:
- 20 the replacement amino acid for residue #28
being further selected from the group
consisting of: Trp, Gly, Pro, Tyr, Phe,
and Met;
- the replacement amino acid for residue #74
25 being further selected from the group
consisting of: Gly, Val, Ile, His, and
Pro;
- the replacement amino acid for residue #76
being further selected from the group
30 consisting of: Gly, Val, Ile, Leu, His,
and Pro;
- the replacement amino acid for residue #119
being further selected from the group
consisting of: Trp, Met, Gly, Pro, Tyr,
35 and Phe;

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- the replacement amino acid for residue #127
being further selected from the group
consisting of: Trp, Gly, Pro, Tyr, and
Phe;
- 5 the replacement amino acid for residue #131
being further selected from the group
consisting of: Trp, Met, Gly, Pro, Tyr,
and Phe;
- 10 the replacement amino acid for residue #134
being further selected from the group
consisting of: Gly, Val, Ile, Leu, and
Pro;
- 15 the replacement amino acid for residue #136
being further selected from the group
consisting of: Trp, Tyr, Phe, Cys, Met,
Lys, Glu, Arg, Gln, Asp, and Asn;
- the replacement amino acid for residue #169
being further selected from the group
consisting of: Gly, Val, His, and Pro;
20 and
- the replacement amino acid for residue #171
being further selected from the group
consisting of: Gly, Val, Met, Ile, and
His.
- 25
21. A modified form of TNF- β as described in claim 20
wherein:
- 30 in said Step E: determining whether the candidate
form of TNF- β has a channel activity that is
materially reduced as compared to the
corresponding channel activity of unmodified TNF- β
and that is employable for reducing the channel
activity of the target membrane; and
- 35 in said Step F: designating as the modified form of
TNF- β a candidate form of TNF- β that is
determined to have a channel activity that is

materially reduced with respect to the corresponding channel activity of unmodified TNF- β .

5

22. A modified form of TNF- β as described in claim 20 wherein:

10 in said Step E: determining whether the candidate form of TNF- β has a channel activity that is materially enhanced as compared to the corresponding channel activity of unmodified TNF- β and that is employable for enhancing the channel activity of the target membrane; and
15 in said Step F: designating as the modified form of TNF- β a candidate form of TNF- β that is determined to have a channel activity that is materially enhanced with respect to the corresponding channel activity of unmodified TNF- β .

20

23. A modified form of TNF- β comprising:

a trimer of three molecules of modified TNF- β , each molecule of modified TNF- β within said trimer being covalently bonded to the other two
25 molecules therein by means of disulfide bonds, for holding said trimer together.

24. A modified form of TNF- β as described in claim 23 wherein:

30 the disulfide bond is formed under non-reducing conditions by cysteine molecules introduced by substitution into the modified TNF- β molecule, the cysteine substitutions being introduced into residues selected from the group consisting of
35 the following sequence numbers:

the pair of residues #119 and #131; the
pair of residues #117 and #135; the
pair of residues #28 and #171; and the
single residue #134; the sequence
5 numbers being defined with respect to
unmodified forms of human TNF- β .

25. A method for regulating the permeability of a TNF
target membrane, the method comprising:

10 contacting the target membrane with a modified form
of TNF, the modified form of TNF having a
reconstructed channel, as compared to unmodified
TNF, for regulating channel activity.

15 26. A method for regulating the permeability of a TNF
target membrane as described in claim 25 wherein:
the modified form of TNF having a reduced channel
activity as compared to the unmodified form of TNF and
the insertion of the modified form of TNF serving to
20 reduce the channel activity exhibited with the target
membrane.

27. A method for regulating the permeability of a TNF
target membrane as described in claim 25 wherein:
25 the modified form of TNF having an enhanced channel
activity as compared to the unmodified form of TNF and
the insertion of the modified form of TNF serving to
enhance the channel activity exhibited with the target
membrane.

30 28. A method for inhibiting the binding of unmodified TNF
to one or more TNF receptors attached to a target membrane,
the method employing a modified form of TNF having a
reconstructed channel for reducing channel activity within
35 a target membrane, the method comprising:

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5 contacting the modified form of TNF with one or
 more of the TNF receptors under conditions for
 permitting binding between the modified form of
 TNF and the TNF receptor, the modified form of
 TNF having a reconstructed channel, as compared
 to unmodified TNF, for reducing channel activity.